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The Action of Oestrin on the Bitch

By J. M. ROBSON (Beit Memorial Research Fellow) and W. R. HENDERSON (from the Institute of Animal Genetics and the Department of Surgery, University of Edinburgh)

(*Communicated by F. H. A. Marshall, F.R.S.—Received April* 27, *Revised November* 29, 1935)

[PLATES 1–3]

The sex cycles in the bitch and in the Primates are both characterized by the occurrence of periodic bleeding from the uterine endometrium. But while in the bitch this bleeding is observed during the time of pro-oestrus, and ovulation and the formation of the corpus luteum occur shortly after its cessation, in the Primates, on the other hand, ovulation and corpus luteum formation take place some time before the menstrual bleeding, the rupture of the follicle occurring about 10–15 days after the beginning of the previous menstrual flow; indeed the luteal activity normally plays an important part in determining those changes in the uterine endometrium characteristic of the premenstrual condition. Moreover, the menstrual bleeding is generally preceded by degeneration of the corpus luteum, though there is good evidence to show that, occasionally, menstruation may occur without any luteal activity whatever.

In view of these facts it is generally agreed (*see* Corner, 1933) that menstruation in the Primates and the pro-oestrous bleeding in the bitch are not homologous phenomena and it has been suggested that pro-oestrous bleeding is the equivalent of the intermenstrual bleeding which has been described as occurring in the Primates (Hartman, 1930; Papanicolaou, 1933; Hain, 1934). Moreover, recent work on the hormonic basis of these various changes has yielded results which need careful examination. In the first place it seems very likely that the pro-oestrous alterations in the bitch are to a great extent dependent on the activity of the oestrous hormone (Asdell and Marshall, 1927; Meyer and Saiki, 1931). And secondly, the recent work on the action of oestrin in the Primates including the human has emphasized the importance of this hormone in connexion with the causation of menstrual bleeding. For not only may uterine bleeding occur in the complete absence of the luteal secretion (though

the majority of observers are agreed that the corpus luteum activity is part of the normal physiological process), but the injection of oestrin into ovariectomized subjects and monkeys is capable of bringing about uterine bleeding, both during the period of administration and after its cessation (Kaufman, 1933; Werner and Collier, 1934; Hisaw, 1935). Thus Werner and Collier (1934) obtained uterine bleeding during the administration of 400 R.U. daily to ovariectomized patients. Kaufman (1933), treating cases of primary amenorrhoea with hypoplastic genitals, also produced bleeding during the administration of the hormone which was given in doses of some 100,000 M.U. per week; and lastly, Hisaw (1935) reports bleeding induced in castrated *Macacus rhesus* during the injection of 40 or 80 R.U. per day. It is therefore of special interest to note that Meyer and Saiki (1931) have advanced experimental evidence (including the finding that bleeding occurred only during the period of oestrin injections) suggesting that the reactions of the bitch and of the Primates to the administration of oestrin are essentially different in nature.

The object of the present investigation was to determine the effect of oestrin on the uterus of the bitch; in particular it was desired to establish whether experimental bleeding produced by the hormone would occur during the period of administration or after its cessation, or both. Some of the animals were hypophysectomized in order that any influence of the pituitary in the bleeding produced might be excluded. This, moreover, also made it possible to investigate whether the oestrous hormone is effective in the absence of the pituitary, as has already been demonstrated for several other species (Hill and Parkes, 1933; Witschi and Levine, 1934; Robson, 1935; Asdell and Seidenstein, 1935).

Technique

The experiments were performed on eight mature bitches. None of them was on heat at the beginning of the experiment, but one of them (D6) was pseudo-pregnant, as shown by the subsequent histological examination of the uterus and ovaries. The animals were ovariectomized by the dorsal route and hypophysectomized by the temporal approach. As was shown by serial sections, hypophysectomy was complete in all animals in which the pituitary was removed, except in D7 in which a small portion of the gland (chiefly posterior lobe cells) was left. At certain stages laparotomies were performed and pieces of uterus removed for examination.

In a number of experiments the *in vitro* activity of the uterine muscle removed was also determined and for this purpose the strips were sus-

pended in Ringer Locke solution at 37·5° C with constant oxygen bubbling and records taken on smoked drums.

Crystalline trihydroxyoestrin and ketohydroxyoestrin (B.D.H.) dissolved in olive oil and a purified preparation of the pituitary oxytocic hormone "Pitocin" kindly supplied by Parke, Davis & Co. were used in these experiments. Animals D1, D2, and D3 were injected with trihydroxyoestrin; D5 received 1·5 mg of trihydroxyoestrin followed by 3 mg of ketohydroxyoestrin while the other bitches received the ketohydroxyoestrin compound only.

Results

The uterus in animals hypophysectomized for some time was small and comparatively avascular, but similar in appearance to the organ in the anoestrous animal. This is illustrated by sections of the uterus removed from animal D2, 17 days after hypophysectomy, from animal D4, 14 days after hypophysectomy and from D3, in anoestrus with the pituitary intact.

The injection of oestrin into the bitch brings about alterations involving (1) the vulva, (2) the vagina, (3) the uterine muscle and endometrium, and (4) the mating reflexes. All these changes (including bleeding from the endometrium) were observed in hypophysectomized as well as in the ovariectomized animals.

The Vulva—Swelling of the vulva began to be noticed on about the 10th day of the injection period, and then increased rapidly until in a few days an oedematous appearance of the same type as that occurring at pro-oestrus was observed. The extent of this swelling, which varied in different animals, was, however, never quite as marked as that observed in the oestrous bitch.

The Vagina—Fluid was withdrawn from the vagina in order to determine whether any erythrocytes were present and the cellular condition was examined. As was found by Evans and Cole (1931), we observed a certain variability in the cellular content at any particular period. In the ovariectomized or hyophysectomized animals investigated the smear consisted mainly of epithelial cells and leucocytes, but following injection of oestrin cornified cells made their appearance and ultimately the smear consisted of cornified cells and epithelial cells but no leucocytes. When bleeding occurred during a period of oestrin injections there was no change in the smear (apart from the presence of erythrocytes) but following

cessation of oestrin administration the vaginal fluid contained an increasing number of leucocytes.

The Uterus—The injections of oestrin produced a marked hypertrophy of the uterus which involved both the endometrium and the muscle and the appearance of the organ became very similar to that obtaining during pro-oestrus. This is illustrated in fig. 2, Plate 1.

In all animals injected for more than 10 days some bleeding occurred (for details, *see* Table I) though its extent varied considerably in different animals. It must be emphasized that the bleeding occurred not only in the ovariectomized animal, but also in the hypophysectomized bitches. In a number of cases the haemorrhage was fairly profuse and lasted for several days, though the loss of blood was always appreciably less than that usually witnessed at pro-oestrus under normal conditions. In some experiments no marked external bleeding occurred but examination of the vaginal fluid under the microscope showed the presence of a large number of erythrocytes. An important feature of this bleeding was that it occurred not only after cessation of the oestrin administration but also during the course of the injections. Indeed, a single series of injections might bring about bleeding both during its administration and after its cessation. Thus in bitch D8 injected for 23 days following bilateral ovariectomy, the vaginal smear contained numerous erythrocytes between the 14th and 18th days of the injection period, while definite external bleeding was observed from the 28th to the 31st days (*i.e.*, 5–8 days after cessation of the injections). In the hypophysectomized animal D5 bleeding was observed only after cessation of the injections (9-day period), while in animal D7 quite profuse bleeding occurred during one injection period and also after another series of oestrin injections. This bitch was initially ovariectomized and then hypophysectomized a considerable time afterwards, but before removal of the pituitary it received two series of oestrin injections, in both of which the daily dose of the hormone was the same. The first period was followed by uterine bleeding but during the second period, lasting 23 days, no bleeding occurred. Following hypophysectomy another series of injections was given and again external bleeding occurred during the period of oestrin administration.

In three cases we removed uterine pieces from animals while external bleeding was taking place or while blood was present in the uterine lumen, and examined the endometrium to determine how the blood passes into the lumen. The subepithelial haemorrhages described by Evans and Cole (1931) were quite evident, and in some cases these extended through small patches of degenerated epithelium into the lumen. This is illus-

TABLE I

Showing the injections given and the effects on uterine bleeding and on mating. The operation is counted as the first day of the experiment except in those animals where the ovaries or pituitary were not removed when the experiment is dated from the first oestrin injection. (2) Small piece of pituitary left (chiefly posterior lobe cells).

Animal No.	Operation	Oestrin injections: Duration days	Oestrin injections: Total amount mg	Period of occurrence with reference to beginning of experiment of: Oestrin injections	Uterine bleeding	Mating	Remarks
D1	Hypophysectomy	10	7·6	10–19	None	—	Experiment ceased on 19th day
D2	,, ...	11	5·3	16–26	*	—	* Blood in lumen of uterus on 27th day
D3	—	10	5·5	1–10	None	—	Experiment ceased on 10th day
D5	Hypophysectomy	9	4·5	7–15	21–23	—	Animal killed on 23rd day
D6	—	13	6·5	1–13	22–23	—	Metoestrous animal. Animal killed on 23rd day
D7	Bilat. ovar. Hypophysectomy (2) on 88th day of experiment	14	7·0	2–15	16–18	17–21	Mating not tried after 39th day during injection period
		23	11·2	34–56	None	37–39	
		15	7·7	105–119	113–118	117–120	
D8	Bilat. ovar.	23	11·2	1–23	14–18 (1) 28–31	—	(1) Microscopic bleeding

trated in fig. 5, Plate 3, obtained from animal D7 on the third day of bleeding during a period of oestrin injections.

The experiment on animal D6 was performed on a bitch in early metoestrus (pseudo-pregnancy) as was shown by the piece of uterus removed before any hormone administration, fig. 3*a*, Plate 2. Injections were given for 13 days, the animal receiving 0·5 mg of ketohydroxy-oestrin daily. No bleeding occurred during that period. On the day following the last injection another piece of uterus was removed, the organ being markedly hypertrophied. Eight days later bleeding was observed and this became profuse on the next day, when the animal was killed. The uterus and ovaries were kept for examination.

The uterine piece removed before the injections shows marked proliferation of the glandular elements in the deeper parts of the endometrium with crypt formation at the surface, fig. 3*a*, Plate 2, a condition characteristic of the height of the metoestrous development. The glands are lined with columnar epithelium. It is unlikely that at that stage of the experiment the animal was more than 14 days post-oestrus. In the piece removed at the end of the oestrin injection period, fig. 3*b*, Plate 2, the glandular development is much less marked and the epithelium lining the glands has now become considerably flatter. The crypts are still well marked and a large number of them are almost filled with structureless material, probably secretory products. The glands in the uterus removed 9 days following the cessation of oestrin injections, fig. 3*c*, Plate 2, and during profuse haemorrhage are lined with flattened epithelium. The crypts are numerous and some of them very large. Structureless material fills some of them, but occasionally this material has sufficient structure to suggest that it originated, to some extent at least, from erythrocytes. The crypts also contain numerous polymorph leucocytes, either alone or in combination with the structureless material. In a number of places the superficial epithelium appears to be breaking down. No diapedesis could be observed in any of the sections.

Mating—In two animals careful observations of the behaviour with the male at various stages of the experiment were made and in bitch D7 mating was observed not only after the ovaries had been excised but also after removal of the pituitary. Moreover there was a definite relation between the occurrence of the mating reaction and the oestrin injections. During the first bleeding following the hormone administration mating took place and this was again observed towards the end of the second period of oestrin injections, though no bleeding apparently occurred. Following hypophysectomy a third period of hormone administration

produced further uterine bleeding and the mating reaction was again observed and indeed continued for some time after cessation of the injections and of bleeding, Table I. In between the injection periods the animal showed not the slightest sexual interest in the male. In order to illustrate the various alterations in the sexual organs during the course of the experiment in this animal an abridged protocol is given in Table II. Similar observations were made in animal D8 but no mating occurred at any stage of the experiment. Towards the end of the oestrin injection period the male attempted to mount the bitch but was always pushed off.

TABLE II

Protocol of experiment on animal D7, showing the effects of oestrin injections before and after hypophysectomy on the vulva, uterine bleeding, and the mating reaction.

Day of experiment	Oestrin injections mg	Bleeding	Mating	Vulva	Remarks
1	—	—	—	—	Bilateral ovariectomy
2–15	0·5 (daily)	—	—	—	—
16	—	—	—	Swelling	—
17	—	Bleeding	Mating	—	—
18	—	,,	,,	—	—
19	—	,,	,,	—	—
20	—	—	,,	—	—
21	—	—	,,	—	—
—	—	—	No mating	—	—
34	0·2	—	—	—	—
35	0·5	—	—	—	—
36	0·5	—	No mating	—	—
37	0·5	—	—	—	—
38	0·5	—	—	—	—
39	0·5	—	—	—	—
40	0·5	—	—	—	—
41	0·5	None	—	—	—
42	0·5	—	—	—	—
43	0·5	—	—	—	—
—	0·5	—	—	Marked swelling	—
50	0·5	—	Mating	—	—
51	0·5	—	—	—	—
—	0·5	—	—	—	—
56	0·5	—	—	—	—
—	—	—	—	—	—
75	—	None	—	—	—

TABLE II—(continued)

Day of experiment	Oestrin injections mg	Bleeding	Mating	Vulva	Remarks
—	—	—	—	—	—
79	—	—	—	—	—
—	—	—	No mating	—	—
82	—	—	—	—	—
88	—	—	—	—	Hypophysectomy
—	—	—	—	—	—
105	0·5	—	—	—	—
106	0·5	—	—	—	—
107	0·5	—	—	—	—
108	0·5	—	No mating	—	—
109	0·5	—	—	—	—
110	0·5	—	—	—	—
111	0·3 M & E	—	—	—	—
112	,,	—	—	—	—
113	,,	Bleeding	—	—	—
114	,,	,,	—	—	—
115	0·3	,,	—	Swelling	Uterine piece removed
116	0·6	,,	—	—	—
117	0·6	,,	Mating	—	—
118	0·3	,,	,,	—	—
119	0·5	—	,,	—	—
120	—	—	,,	—	—
121	—	—	,,	—	—

Reactivity to Oxytocin—The *in vitro* reactivity to oxytocin was determined on the uterine strips removed at various stages of the experiment and the results are given in Table III; the reactivity is expressed as the minimum dose per 100 cc of solution capable of eliciting a motor effect. In the first six animals the reactivity was determined before the administration of oestrin began and also immediately after the injection period, while in the last two animals no such observations previous to the hormone injections were made. In addition, in animals D5 and D6, determinations were again made some time after the cessation of oestrin injections. It will be seen that, following the removal of the ovaries or of the pituitary, the uterine reactivity remains comparatively high, the minimal effective dose of oxytocin varying from 0·01 to 0·03 units per 100 cc of solution. For example the uterus of animal D2, 16 days after hypophysectomy, showed an *in vitro* response to 0·02 units of the posterior lobe hormone per 100 cc of Ringer Locke. Moreover the administration of oestrin does not appear to cause any increase in the reactivity to oxytocin. On

TABLE III

Showing the *in vitro* reactivity to oxytocin of the uterus at various stages of the experiments. In animals 1–6 the reactivity was measured both before and after the oestrin injections while in 7 and 8 no determinations previous to the injections were made. In animals 5 and 6 determinations were made not only immediately after cessation of injections but also at a subsequent period.

Animal No.	Operation	Interval between operation and determination of reactivity Days	Reactivity before injections Units per 100 cc	Oestrin injections		Interval between 1st and 2nd determinations of reactivity Days	Reactivity after injections Units per 100 cc	Interval between 1st and 3rd determinations of reactivity	Reactivity Units per 100 cc	Remarks
				Duration Days	Total amount mg					
D1	Hypophysectomy ..	10	0·03	10	7·6	10	1·0	—	—	—
D2	,, ..	16	0·02	11	5·3	12	0·02	—	—	—
D3	—	—	0·002	10	5·5	10	0·01	—	—	—
D4	Hypophysectomy ..	14	0·01	—	—	—	—	—	—	—
D5	,, ..	7	0·01	9	4·5	11	0·05	17	0·03	Bleeding at 2nd determination of reactivity
D6	—	—	0·05	13	6·5	16	0·05	25	0·02	,, ,,
D7	Bilat. ovar.	115	—	—	—	—	—	—	—	Bleeding at determination of reactivity
	Hypophysectomy ..	27	—	10	5·7	—	>0·1	—	—	
D8	Bilat. ovar.	23	—	23	11·2	—	0·02	—	—	—

the contrary the data rather suggest that the minimum effective dose of the oxytocic hormone is increased immediately after a period of oestrin injections. This was especially so in animal D1 in which 0·03 units of "Pitocin" caused a contraction before the administration of oestrin was started while, after the injection period, 1·0 unit of posterior lobe hormone had to be added to the bath in order to elicit a motor effect.

Discussion

The results of these experiments indicate that the oestrous hormone may bring about in the bitch qualitative alterations similar to those occurring during pro-oestrus and oestrus. Not only are the external genitalia and the vagina affected but histological changes, resembling closely those present during pro-oestrus, are produced in the uterus and bleeding may occur from that organ. Moreover the mating reflexes may be activated under the influence of oestrin. With the exception of mating all these changes were brought about in the completely hypophysectomized animal and it thus appears probable that the pituitary is not essential for their occurrence. In the animal in which mating was produced (D7) subsequent examination showed that a very small portion of the gland (chiefly posterior lobe cells) had been left behind at the operation, so that we cannot be certain that some pituitary activity may not have been concerned in the mating reaction which was elicited.

The examination of the uterus during the period of bleeding experimentally induced probably offers an explanation for the mechanism of the bleeding, for it was possible to demonstrate definitely the occurrence of the passage of erythrocytes into the lumen in two cases. Evans and Cole (1931) indeed suggested that this mechanism might be responsible but were unable to find any evidence of it in the material they investigated. In the present experiments, moreover, there was also evidence of slight degenerative changes in the superficial part of the endometrium from which bleeding was taking place, an observation similar to that described by Marshall and Jolly (1905) in the pro-oestrous animal.

The question now arises as to what sequence of hormonic effects is responsible for the alterations observed during the normal oestrous cycle in the bitch and what relation do these changes bear to those occurring during the menstrual cycle in the Primates. In the first place it appears likely that the typical histological changes occurring at pro-oestrus are dependent upon the action of oestrin. Moreover the quantities of hormone produced under normal conditions must be considerable, as the comparatively large doses we administered did not produce a degree

of swelling of the external genitals equal to that observed under physiological conditions. No definite answer can at present be given as to the temporal relation between the period of oestrin secretion and the events of the oestrous cycle and more especially bleeding. Meyer and Saiki (1931) who injected oestrin preparations into castrated bitches found that bleeding always occurred *during* the period of administration, and as this is not so in the Primates, they concluded that the mechanism producing uterine bleeding in the bitch and in the Primates cannot be similar. On the other hand we obtained bleeding not only during but also after the cessation of oestrin injections and at periods varying from 4 to 8 days after the last administration of the hormone. This discrepancy is quite possibly due to differences in the doses given and as the amounts of oestrin injected in the present experiments were apparently larger than those used by Meyer and Saiki (the data do not unfortunately permit of an accurate comparison) it seems possible that, under physiological conditions, uterine bleeding may only ensue after a diminution of the high rate of secretion responsible for the initial alterations in the uterus, external genitals, etc. It is of interest to note that in the ovariectomized human subject and in *Macacus rhesus* experimental administration of oestrin produces uterine bleeding not only following but also during the injection period (Werner and Collier, 1934; Kaufman, 1933; Hisaw, 1935), while studies on hormone content of the blood and hormone excretion (Zondek, 1931; Siebke, 1930) and on the sexual skin of *Macacus rhesus* (Corner, 1933) strongly suggest that the level of oestrin secretion is high during the period preceding bleeding and low during menstruation.

The results obtained by the authors on the bitch, when compared with those reported by previous workers on Primates, show that in both cases oestrin injections may cause bleeding both during the period of injection and after termination of the injections. In both cases the experimental occurrence of bleeding during the course of oestrin injections still awaits a satisfactory explanation. It is clear, however, that there is no certain difference between the times at which bleeding occurs in the two genera.

Some time after the initiation of bleeding a new hormonic factor is brought into play, following ovulation and the formation of the corpus luteum. The events which, under physiological conditions, now ensue are, however, different in the bitch and in the Primates. For the luteal proliferation of the uterus in the bitch is probably not accompanied by any marked oestrin secretion and hence the cessation of luteal activity, either normally or after removal of the ovaries (Meyer and Saiki, 1931), is not followed by profound degenerative changes although slight degeneration may apparently occur (Marshall and Halnan, 1917). In

the Primates, on the other hand, the luteal phase during the menstrual cycle is accompanied by a high level of oestrin secretion for which the corpus luteum itself is probably, to some extent at least, responsible. Hence the fall in the oestrin production which accompanies the end of the luteal secretory phase in the menstrual cycle is followed by the degenerative changes which constitute menstruation.

But it is possible to produce experimentally in the bitch an oestrous phase during the period when the luteal activity is decreasing and under these conditions cessation of oestrin administration is followed by a uterine bleeding. Thus a " telescoping " of these two phases of ovarian activity, suggested by Marshall (1927) as being involved in menstruation in the Primates, has actually been experimentally produced in the bitch.

What then is the relation of ovulation to the period of high oestrin secretion which, both in the bitch and in the Primates, apparently controls uterine bleeding? In time there is no great difference in the intervals between initiation of bleeding and ovulation in the two species. For while in the Primates ovulation occurs some 10–15 days after the beginning of menstruation, it seems probable that in the bitch a mean period of 11 days elapses between the beginning of pro-oestrous bleeding and ovulation (*see* Evans and Cole, 1931, for the data on the bitch). Of course in the bitch the bleeding only decreases shortly before ovulation, while in the Primates this interval between the cessation of bleeding and ovulation is almost invariably more prolonged. It is, however, a moot point whether this latter interval is of greater significance than that which elapses between the *beginning* of bleeding and ovulation and it is at least arguable that the actual duration of the bleeding which follows marked oestrin activity is essentially dependent upon the reaction of the uterine endometrium to a given stimulus, and as such is not of paramount importance in the evaluation of the factors which control the histological and secretory changes in the uterus. That the reaction of the uterus to a given stimulus may show considerable variations is actually demonstrated by animal D7 which showed marked bleeding following the first series of oestrin injections and no bleeding whatever when the same doses were repeated shortly afterwards.

The determinations of uterine reactivity to oxytocin seem to show that in the bitch oestrin does not produce effects similar to those described in the rabbit and in the mouse (Robson, 1933, 1935); on the contrary, under the experimental conditions described, the hormone causes either no effect or a decrease in reactivity to the posterior lobe principle. These results cannot, however, be fruitfully discussed until data for the altera-

tions during the various stages of the sex cycle in the bitch have been obtained.

We wish to express our gratitude to Dr. F. H. A. Marshall, F.R.S., for his valuable advice.

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Summary

Injections of oestrin in the ovariectomized and hypophysectomized bitch bring about alterations in the external genitals, vagina, and uterus similar to those found during pro-oestrus. Mating was induced in one animal in which the pituitary was almost completely removed. Uterine bleeding was observed both during the course of injections and following their cessation, and occurred in the absence of the pituitary. The passage of the erythrocytes into the uterine lumen during experimental bleeding was clearly demonstrated in two animals.

Injections of oestrin into a pseudo-pregnant animal were followed by uterine bleeding, accompanied by marked degenerative changes in the endometrium.

The *in vitro* uterine reactivity to oxytocin following ovariectomy and hypophysectomy, and after a series of oestrin injections, was determined.

The alterations following oestrin injections, and their relation to the normal changes in the bitch and in the Primates during the sex cycle are discussed, and it is pointed out that the evidence presented supports the view that menstruation in the Primates represents a " telescoping " of pseudo-pregnant degeneration and pro-oestrus change into one process.

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Description of Plates

Fig. 1—Illustrating the appearance of the uterus in the anoestrous and in the hypophysectomized bitch. × 30.

a—Anoestrous animal (D3).
b—14 days after hypophysectomy (D4).
c—17 days after hypophysectomy (D2).

Fig. 2—Illustrating the effect of oestrin on the uterus of the hypophysectomized bitch. Section of uterus of animal D2 after oestrin injections over a period of 11 days. × 30. (Compare with fig. 1*c*.)

Fig. 3—Showing the effect of oestrin on the uterus of the pseudo-pregnant animal (D6). × 45.

a—Before the injection period. Note the typical glandular proliferation.
b—On the day after the injection period.
c—During the ensuing period of uterine bleeding. Note the degenerative changes.

Fig. 4—Higher power photograph of fig. 3*c*, illustrating alterations in the endometrium. Note that the epithelium is still present in one part of the section, but not in the adjacent part. × 300.

Fig. 5—Showing the occurrence of the passage of erythrocytes into the lumen during uterine bleeding (animal D7). × 200.

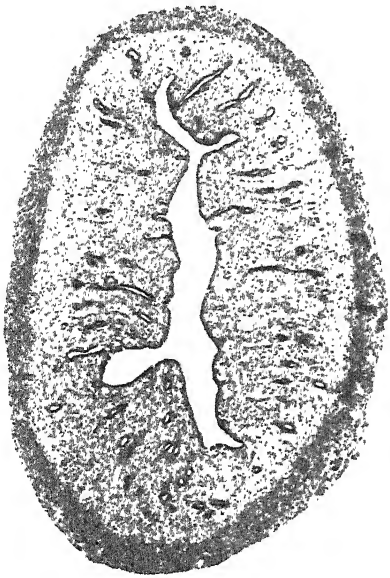

FIG. 1*a*.

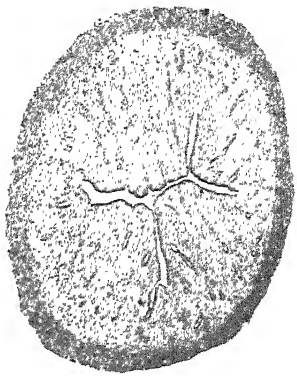

FIG. 1*b*.

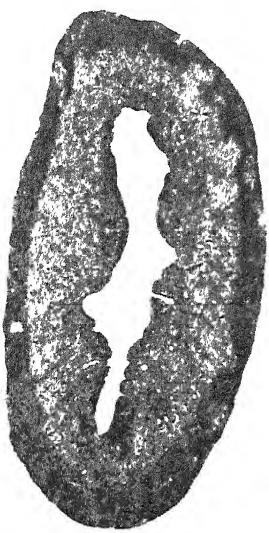

FIG. 1*c*.

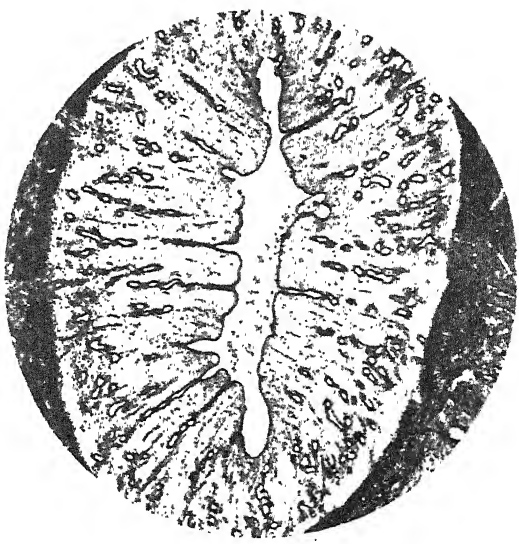

FIG. 2.

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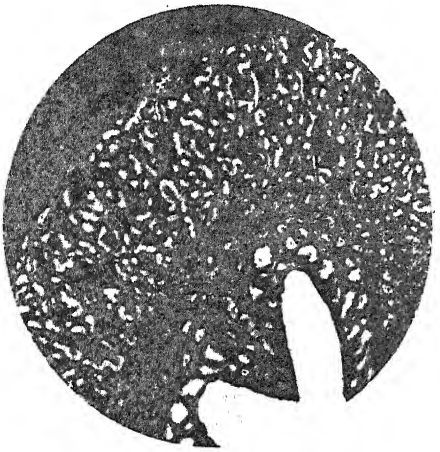

FIG. 3*a*.

FIG. 3*b*.

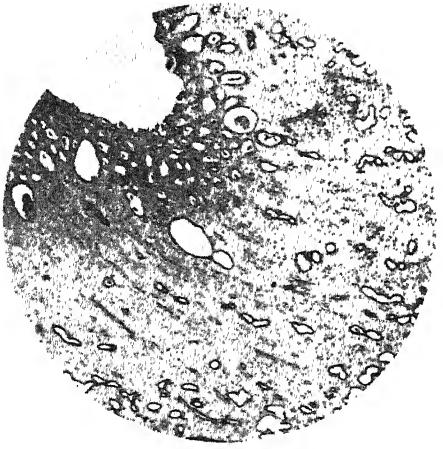

FIG. 3*c*.

FIG. 4.

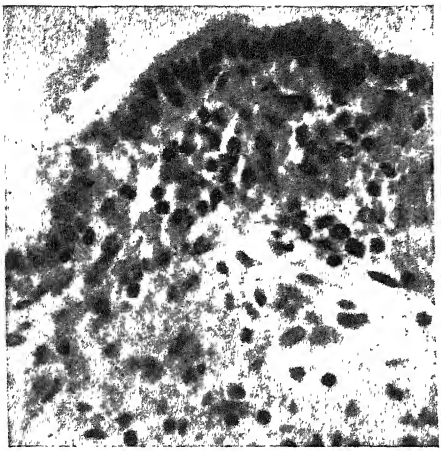

FIG. 5.

611.77 : 539.217

On the Nature and Permeability of Chitin
II—The Permeability of the Uncalcified Chitin Lining the Foregut of *Homarus*

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I—INTRODUCTION

It has been shown (Yonge, 1932) that the integument of the Decapod Crustacea, as exemplified by the uncalcified lining of the foregut of the lobster, *Homarus vulgaris*, consists of two layers which differ widely in nature and origin. There is a thin superficial cuticle which is hyaline, possesses adsorbed lipin, and is formed by the widely distributed tegumental glands the function of which had previously been obscure. The actual chemical nature of this thin layer was not determined but it is *not* chitin from which it can be distinguished by a variety of chemical and physical tests. The underlying and much thicker layer of the integument consists of lamellated chitin formed by the cells of the epithelium. The present research was designed to determine in broad outline the permeability of this membranous integument, and in particular the influence upon this of the bounding cuticle and the general biological significance of the cuticle.

II—LITERATURE

In the Crustacea, Jordan and Lam (1918) found that the foregut and hindgut of *Astacus*, which are lined with chitin, behave as semipermeable membranes, allowing water, but not dissolved substances, either electrolytes or non-electrolytes, to pass through under the influence of osmotic pressure. Similar results were obtained by Yonge (1924) with the foregut of *Nephrops*. Very different results were obtained from similar experiments with the midgut of both *Astacus* and *Nephrops*, indicating that the peculiar properties of the remainder of the gut are due to the chitinous lining. Murlin (1902) and Nicholls (1931) have shown that the chitin which lines the so-called midgut in *Oniscus*, *Porcellio*, and other land Isopoda, and in *Ligia oceanica* respectively, is permeable to the end-

products of digestion. Krogh (1915) states that the gills of *Astacus* are practically impermeable to urethane. Fischel (1908), Koehring (1930, 1931), Gickelhorn (1931), and Bond (1933) have all found some evidence for the penetration of the integument of various Cladocera and Copepoda by vital stains.

In the Insecta, Gorka (1914) found that the chitinized hindgut of Coleoptera was permeable to water. Petrunkevitch (1900) and Sanford (1918) both found that the chitinized crop of the cockroach was permeable to the products of fat digestion, and this has been confirmed by Abbott (1926) who states, however, that water and water soluble substances do not pass through the wall of the crop even after 72 hours and that this, unlike the colon and rectum—both chitinized—does not behave as a semipermeable membrane. Eidmann (1922), who also worked on cockroaches, found that thin chitin behaved as a semipermeable membrane and that potassium hydroxide penetrated through it, quickly through the thin chitin of the colon and very slowly through the thicker chitin from the crop. He concluded that the permeability of chitin depends on its thickness. Wigglesworth (1929) states that the peritrophic membrane in the Tsetse-fly, *Glossina*, is " permeable to colloidal particles of the magnitude of the haemoglobin molecule and also to the proteolytic enzyme ". Wigglesworth's theories as to the nature of tracheal respiration in Insecta (1930) and on the function of the rectal glands (1932) postulate the permeability to water of the chitin in the trachea and rectum respectively. His observations on the effect of salts on the anal gills of mosquito larvae (1933, *a*) have also certain bearings on chitin permeability.

Krogh (1919) in a study of the rate of diffusion of gases through animal tissues found that the absolute constant for the passage of oxygen at 20° C through chitin was 0·013, compared with 0·34 for water, 0·28 for gelatine, 0·14 for muscle, 0·115 for connective tissue, and 0·077 for india-rubber. Chitin was thus much less permeable to oxygen than other tissues or even india-rubber.

III—Material and Methods

Lobsters were opened and the foregut removed, slit open along the midventral line and the contents washed out. After soaking in running water overnight the macerated tissues peeled off leaving the integument which, after further washing, was ready for use. The " pyloric " (posterior) portion of the " stomach " and also the gastric mill at the posterior end of the " cardiac " portion, were cut away leaving a roughly circular area of uncalcified integument some 4 cm in diameter in an average sized lobster.

The technique employed, though simple, gave consistent results. Glass tubing, *t*, fig. 1, with an internal diameter of from 2 to 2·5 cm was cut in lengths of about 7·5 cm and a flange, *f*, made at one end. Pieces of the membrane, *m*, were securely fastened round this end with a binding of stout thread, *th*. The membrane is exceedingly tough and this method of attachment proved admirable, only very rarely did membranes rupture during attachment while the joint was invariably water-tight. The membranes were fastened sometimes with the original free surface (bounded with cuticle, *see* Yonge, 1932) to the outside and sometimes to

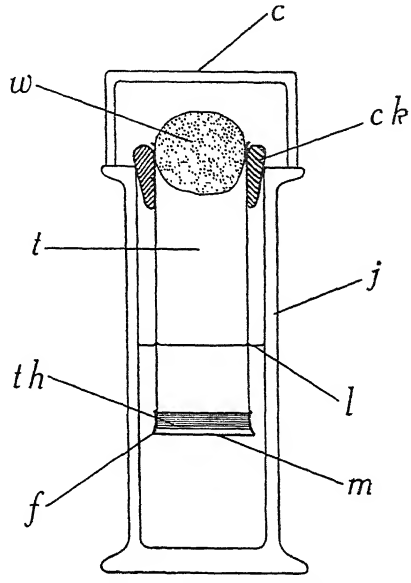

FIG. 1—Apparatus employed in experiments, for interpretation of lettering *see* text. × ½.

the inside. The tubes were numbered and the two series marked " A " and " B " respectively.

Experiments were conducted by fitting these tubes inside round glass jars, *j*. Into the jars were measured 40 cc of some liquid (usually distilled water), the tubes were then inserted and retained temporarily in position above the level of the liquid by wedges of cork, *ck*. The jars were placed in a thermostat maintained at a constant temperature of 20° C. A definite quantity (usually 10 cc) of liquid was quickly pipetted into the tubes which were then pushed down until the levels of the liquids inside and out corresponded, as shown in fig. 1, *l*. A wad of cotton wool, *w*, was inserted into the open end of the tube and finally a glass cover, *c*, placed over the flattened lips of the jar. After definite periods the jars

were removed, the tubes withdrawn above the level of the liquid in the jars, and the liquid adhering to their outer surfaces quickly washed into the jars with a jet of water from a wash-bottle. The tubes were then removed and washed. They were kept in *running tap water* between experiments; if this were not done they quickly rotted (even in toluol water), although in running water they can be kept almost indefinitely.

Finally the amount of the experimental solute which had passed through the membrane in the given period was determined by estimating the amount present in the water in the jars. Acids and alkalies were titrated, chlorides were estimated with silver nitrate, and glucose by the Hagedorn and Jensen method. The purest chemicals obtainable were always used.

Repetition of experiments proved that the error in this procedure was very small, always very much less than the difference between the figures for different experiments. At the same time it must be emphasized that the results obtained are *comparative only*. Not only did the tubes vary slightly in diameter and particularly in the extent of the flange, but the speed of passage naturally decreased continually as the solutes passed from a region of originally high concentration to one of low concentration. Actually with fresh membranes the amount of solute which passed was usually so slight as to render this factor negligible, but this was not so for treated membranes (*see* below). Although absolute values were not obtainable, comparative values, *i.e.*, the *relative speeds* at which substances passed through the membranes, were clearly demonstrated.

Before proceeding to a description of the experiments it must be understood that, whatever its other properties, the fresh membrane invariably allows water to pass freely through it in either direction under the influence of osmotic pressure, although it is not, as will be shown, a strictly semi-permeable membrane.

IV—Passage of Fatty Acids through Fresh Membranes

Previous statements that the products of fat digestion penetrate the intima of the crop of the cockroach led to a study of the relative rates of passage of the lower fatty acids and of hydrochloric acid. Similar results were obtained with both " A " and " B " membranes. The results of a series of experiments are shown graphically in fig. 2.

Fatty acids penetrate fresh membranes much more rapidly than does hydrochloric acid, only about 2% of which passed through during the experimental period (2 hours). In order of speed of passage the acids arranged themselves in the series:—formic > propionic > butyric > acetic > hydrochloric. The peculiar arrangement of the fatty acids may

be explained on the assumption that there is a tendency for these to pass through the membrane more rapidly with increasing molecular weight but that this is counterbalanced, somewhat irregularly, by the effect of the accompanying decrease in degree of dissociation.

Previous histological study of the membrane (Yonge, 1932) led to the discovery of the cuticle with its adsorbed lipin. Clearly if the presence of this substance is responsible, as was at once suspected, for the greater permeability of the membrane to fatty acids than to mineral acids, permeability will be fundamentally altered if this is removed.

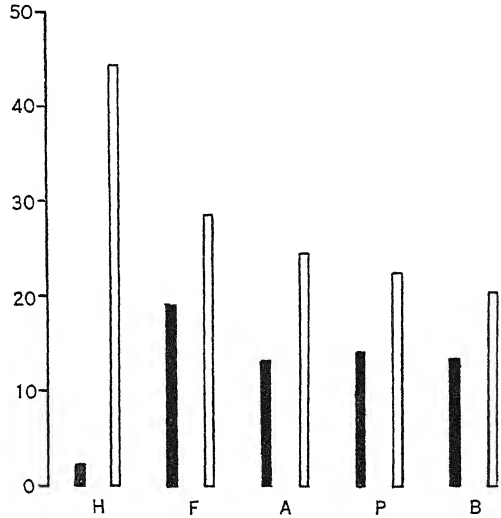

FIG. 2—Diagram showing relative rates of passage of 0·1N hydrochloric, H, formic, F, acetic, A, propionic, P, and butyric, B, acids through fresh and treated membranes (former shown black). Ordinates represent per cent of acid passing through the membrane. Each column represents the average for four experiments. Times, 2 hours for fresh and 1 hour for treated membranes.

V—PREPARATION OF TREATED MEMBRANES

As already recorded (Yonge, 1932), the lipoid substance as revealed histologically by Ciaccio's method, can be partially removed by treating the membrane with fat solvents, alcohol, ether or chloroform, or with normal hydrochloric acid or sodium hydroxide which hydrolyse it. Series of experiments were carried out in which membranes already fixed to tubes were treated with these and other liquids for various periods. It was eventually found that the effect of the lipin could only be *completely* destroyed by prolonged immersion (3 to 4 days) in normal solutions of potassium or sodium hydroxide. After this treatment the membranes

were thoroughly washed in running water and then stored in absolute alcohol. It will be convenient to refer to these as *treated membranes* in contradistinction to the *fresh membranes*. Sections of treated membranes revealed that the cuticle was still present but excessively thin and occasionally ruptured. No trace of lipin was revealed by Ciaccio's stain, and the permeability of these membranes was invariably entirely different from that of fresh membranes. There can be little doubt (and additional evidence for this is given in § XII) that after this treatment the cuticle is *effectively* if not actually removed, experiments with treated membranes revealing the permeability of the *chitin* as distinct from that of the cuticle which effectively controls permeability in fresh membranes.

TABLE I—EFFECT OF PROGRESSIVE REMOVAL OF LIPOID SUBSTANCE ON THE PERMEABILITY OF MEMBRANES TO HYDROCHLORIC AND FORMIC ACIDS

10 cc O·1N acid in tubes; 40 cc distilled water in jars; experiments for 1 hour at 20° C

	% acid in tubes passed through membranes											
Tube	HCl	Formic		HCl	Formic		HCl	Formic		HCl	Formic
A_4	1·3	10·2	Methyl acetate 24 hours	4·4	13·4	Ether 24 hours	19·6	17·0	N NaOH 4 days alcohol 3 days	36·0	21·9
A_7	0·6	9·2		2·7	12·0		16·3	15·6		31·8	19·3
B_2	0·6	9·4		3·1	12·0		5·2	10·0		28·0	16·5
B_{10}	0·4	12·1		4·1	15·5		8·3	14·9		35·0	21·3

One series of experiments, summarized in Table I, reveals the effect of progressive removal of lipin on the penetration of hydrochloric and formic acids. The membranes were treated first with methyl acetate, then with ether (after previous dehydration), and finally with normal sodium hydroxide (and later storage in absolute alcohol) which completely destroyed the influence of the cuticle. The methyl acetate increased permeability slightly but for approximately equal amounts in both cases (though proportionately much more for hydrochloric acid). The effect of ether was greatly to increase permeability to hydrochloric acid whereas the passage of formic acid was decreased not only relatively to hydrochloric acid but also, in the two last experiments, *absolutely*. Finally the complete destruction of the effect of the cuticle with its contained lipin led to a reversal of the original permeability of the membrane to the two acids.

Whereas the permeability of different samples of *treated* membranes, *i.e.*, of the *chitin alone*, does not vary significantly, that of different *fresh*

membranes—which is almost certainly conditioned by the amount of lipin actually contained in the cuticle in each individual membrane—varies very greatly. This is shown in Table II which gives details of the permeability to decinormal hydrochloric acid of six membranes, first in the fresh condition and later after treatment. These membranes were attached to specially selected tubes the diameter across the flange being in each case approximately 3·0 cm and the area of the cross-section being therefore 7·07 cm².

TABLE II

Tube	%/cm² of N/10 HCl passed through membrane in 2 hr	
	Fresh membranes	Treated membranes
A_1	0·28	7·6
A_6	0·44	7·7
A_7	0·70	7·4
B_3	0·14	7·6
B_{10}	0·47	7·4
B_{13}	0·33	7·7

Although the percentage of acid which passed through unit area in the fresh membranes varied from 0·14 to 0·70, in the treated membranes the percentage only varied between 7·4 and 7·7. Essentially the same differences were obtained when decinormal sodium or potassium hydroxides or molar solutions of chlorides were used.

VI—PASSAGE OF FATTY ACIDS THROUGH TREATED MEMBRANES

After the experiments recorded in § IV had been conducted the membranes used were treated with potassium hydroxide and a second series of experiments carried out. The experimental period was reduced to 1 hour. The results are shown graphically in fig. 2.

The acids pass through treated membranes strictly in accordance with the degree of dissociation of each, hydrochloric acid, of which between 41 and 51% passed through the membranes, rising from the lowest place in the series to the highest and the fatty acids following in the series formic to butyric.

VII—PASSAGE OF MINERAL ACIDS THROUGH FRESH AND TREATED MEMBRANES

A comparison between the permeability of fresh and treated membranes to the strong mineral acids, hydrochloric, nitric, and sulphuric, shows, as

the experiments summarized in Table III indicate, that although the permeability of treated membranes is invariably much greater, in both series of experiments the acids pass through in the same order, nitric > hydrochloric > sulphuric. The apparent closing up of the series in treated membranes is the result, as already stated, of the greater amount of acid which passes through these membranes during the experimental period with a consequent greater relative slowing down the faster the acids penetrate.

TABLE III—PASSAGE OF MINERAL ACIDS THROUGH FRESH AND TREATED MEMBRANES

10 cc 0·1N acids in tubes; 40 cc distilled water in jars; experiments for 2 hours at 20° C

Tube	% HCl passed	Relative to HCl = 1	
		HNO_3	H_2SO_4
	Fresh membranes		
A_6	3·4	1·26	0·74
A_{15}	2·6	1·33	0·75
B_{10}	3·3	1·32	0·74
B_{12}	1·5	1·25	0·78
	Treated membranes		
A_6	48·8	1·04	0·84
A_{15}	43·0	1·05	0·86
B_{10}	52·5	1·04	0·87
B_{12}	45·5	1·04	0·85

VIII—PASSAGE OF ALKALIES THROUGH FRESH AND TREATED MEMBRANES

The permeability of fresh and treated membranes to the strong alkalies, lithium, sodium, and potassium hydroxides, was investigated and with these ammonium hydroxide—notable as being a fat solvent—and also barium hydroxide as an example of a divalent base. Table IV shows the results of typical experiments. Whereas with both membranes, lithium, sodium, and potassium hydroxides pass through with increasing speed in the order named, both ammonia and barium hydroxide display interesting peculiarities. Although a weak base, ammonia penetrates fresh membranes much more quickly than do the strong alkalies, but

there is *no definite relation* between the speed of its passage and that of lithium, sodium, and potassium hydroxides, which in all cases have practically the same relations to one another. The great speed with which ammonia penetrates the membranes seems due to the presence of the lipin and the irregularity of its passage to the varying amounts of this present in different membranes. This confirms and extends the results of the experiments summarized in Table II. With treated membranes ammonia drops to the bottom of the series, potassium—sodium—

TABLE IV—PASSAGE OF ALKALIES THROUGH FRESH AND TREATED MEMBRANES

0·1N alkalies used, procedure as in Table III

Tube	% KOH passed	Relative to KOH = 1			
		LiOH	NH_4OH	NaOH	$Ba(OH)_2$
	Fresh membranes				
A_4	1·9	0·67	8·20	0·84	0·64
A_{12}	2·0	0·69	6·25	0·88	0·63
A_{14}	2·1	0·73	6·10	0·85	0·63
B_3	3·9	0·71	3·90	0·87	0·29
B_4	1·3	0·70	6·25	0·80	0·34
B_{22}	6·1	0·68	2·10	0·88	0·30
	Treated membranes				
A_4	52·3	0·75	0·31	0·88	0·87
A_{12}	42·5	0·73	0·31	0·88	0·83
A_{14}	44·8	0·75	0·30	0·87	0·85
B_3	53·6	0·75	0·28	0·86	0·85
B_4	30·0	0·72	0·28	0·86	0·86
B_{22}	50·5	0·77	0·29	0·86	0·85

lithium—ammonium, because here the speed of penetration is apparently controlled exclusively by the degree of dissociation.

A new problem arises with barium hydroxide. In its passage through treated membranes it occupies a position between lithium and sodium, and all experiments gave similar results. With fresh membranes, however, the relationship between its passage and that of potassium hydroxide through "A" membranes (average 0·63) is about double that through "B" membranes (0·31), *i.e.*, it penetrates the membranes twice as quickly from the chitin side to the cuticle side as it does in the opposite direction. The former corresponds more closely to conditions that prevail with treated membranes, although the speed of passage (again in

relation to that of potassium hydroxide) is distinctly less, barium in this case preceding instead of following lithium in the ascending series. It appears that the presence of the lipin slows down the rate of penetration of a divalent cation but very much more when this has to pass first through the free surface of the cuticle.

IX—Passage of Chlorides through Fresh and Treated Membranes

The greater difficulty with which divalent ions penetrate fresh membranes, especially series "B", was confirmed by experiments on the

Table V—Passage of Chlorides through Fresh and Treated Membranes

10 cc molar solutions used; experiments for 3 hours with fresh and 1 hour with treated membranes

Tube	% KCl passed	Relative to KCl = 1						
		HCl	LiCl	NH_4Cl	NaCl	$MgCl_2$	$CaCl_2$	$CdCl_2$
	Fresh membranes							
A_6	1·48	4·28	0·64	1·24	0·80	0·18	0·21	0·09
A_{10}	1·08	6·33	0·65	1·28	0·84	0·18	0·22	0·09
					Average =	0·18	0·22	0·09
B_3	1·08	9·68	0·69	1·28	0·83	0·10	0·12	0·07
B_{10}	0·63	8·50	0·67	1·23	0·81	0·11	0·12	0·08
	Average =	—	0·66	1·26	0·82	0·11	0·12	0·075
	Treated membranes							
A_4	28·2	1·44	0·73	0·96	0·84	0·65	0·70	0·55
A_6	33·7	1·34	0·74	0·94	0·86	0·68	0·73	0·55
					Average =	0·67	0·72	0·55
B_{10}	31·1	1·40	0·77	0·96	0·88	0·62	0·69	0·55
B_{13}	35·6	1·39	0·75	0·93	0·86	0·67	0·70	0·56
	Average =	1·39	0·75	0·95	0·86	0·65	0·70	0·56

passage of chlorides of the monovalent ions, Li, NH_4, Na, K, and H, and of the divalent ions Ca, Mg, and Cd. Molar solutions were employed. The results of a typical series of experiments are given in Table V.

Analysis of Table V shows that:

1—The chlorides of the alkali metals penetrate both fresh and treated membranes in the ascending series lithium > sodium > potassium, as did the hydroxides of these metals. This agrees with the known mobilities of the cations, Table VI.

2—Ammonium chloride penetrates the fresh membranes at a *consistently* greater speed than potassium chloride (in contrast with the irregular relations of speed of passage of the hydroxides), and at a *consistently* slightly slower speed through treated membranes. The latter is in agreement with the mobility of NH_4, Table VI. The former cannot, in view of its consistency in relation to potassium chloride, be due to the lipin (which varies) and is possibly due to the more acid nature of the ammonium chloride solution. The effect of hydrogen ion concentration on the membranes is discussed in § X.

TABLE VI

Solution	Passage in relation to that of KCl at 20° C through			Ionic mobility of cations at 18° C
	Fresh membranes "A"	Fresh membranes "B"	Treated membranes	
$CdCl_2$	0·09	0·075	0·56	0·37
$MgCl_2$	0·18	0·11	0·65	0·36
$CaCl_2$	0·22	0·12	0·70	0·40
LiCl	0·66		0·75	0·52
NaCl	0·82		0·86	0·67
NH_4Cl	1·26		0·95	0·99
KCl	1·00		1·00	1·00
HCl	irregular		1·39	4·93

3—Hydrochloric acid penetrates the fresh membranes much more rapidly than potassium chloride but quite irregularly, *i.e.*, its passage must be influenced by the varying amounts of lipoid substance present in different membranes. In comparison with potassium chloride, hydrochloric acid penetrates treated membranes appreciably more rapidly, and the relationship between the two is consistent. Probably hydrochloric acid at this high concentration (molar) is assisted in its passage by some hydrolysis of the lipin, the fresh membranes being thereby partially converted into treated ones. The acid was the last substance used in the experiments on fresh membranes, and experiments showed that after its passage the permeability of fresh membranes to potassium chloride altered appreciably. The passage of the acid through

treated membranes is in accordance with the high mobility of the hydrogen ion, Table VI.

4—The divalent cations with the exception of cadmium (*see* next paragraph) follow the natural series, Table VI, in their passage through treated membranes. With fresh membranes they behave as did barium in the hydroxide series. Their rate of penetration was much slower in comparison to that of potassium chloride than it was with the treated membranes, and they also penetrated " A " membranes appreciably more quickly than " B ", whereas with treated membranes there was no significant difference between the two. Clowes (1916) originally drew attention to the probable significance of the monovalent/divalent cation ratio in the maintenance of the cell-membrane. On the assumption that this consists of an emulsoid system, the two phases being lipin and a watery solution of proteins, an excess of divalent cations will cause the lipoid phase to become external and continuous, while with an excess of monovalent cations the watery protein phase will be external. In the former case permeability to water-soluble substances will be reduced, in the latter increased. This theory has received substantial support from the work of Dixon and Bennet-Clark (1930, 1932) on the electrical properties of oil-water emulsions and their resemblance to those of plasmatic membranes. A similar explanation will account for the slower relative rate of passage of divalent cations through fresh membranes. The difference in the speed of passage through " A " and " B " membranes indicates that the nature of the interface cuticle-chitin is different from that of the outer surface cuticle-water.

5—MacLean and MacLean (1927) state that lecithin forms addition compounds with certain salts of heavy metals, lecithin-cadmium-chloride being one of these. The exceptionally slow rate of passage of $CdCl_2$ through fresh membranes and also the fact that after its passage the membranes invariably had an opaque white appearance (normally they are practically colourless) may both be accounted for on the assumption that such an addition compound is formed. These facts, together with those discussed in the preceding paragraph, provide further confirmation for the view (Yonge, 1932) that the lipoid constituent in the cuticle is either lecithin or else some very closely allied substance.

In Table VIII the average figures for the penetration of fresh membranes (both " A " and " B " for divalent cations) and of treated membranes are compared with the known mobilities of these cations in water, the figures for the latter being taken from Kaye and Laby (1921). The effect of the lipoid constituent in the cuticle on the permeability of the fresh membrane is strikingly demonstrated when the figures for fresh and

treated membranes are compared. Those for treated membranes agree with those for the ionic mobilities with the exception of cadmium, and here the possibility that it forms an addition compound with the lipin in fresh membranes and the lower hydrogen ion concentration of the solution in the treated membranes may between them account for the discrepancy. In these comparisons again it is the *relationship* between the various figures and *not their absolute values* which is significant.

X—Influence of Hydrogen Ion Concentration on the Passage of Glucose through Fresh and Treated Membranes

Non-electrolytes, such as glucose or glycerol, penetrate fresh and treated membranes. Advantage was taken of this to determine the influence of hydrogen ion concentration on the permeability of both types of membrane. Glucose was employed owing to the ease with which it can be estimated. The buffer solutions of McIlvaine (p_H 2·2–8·0) and of Clark and Lubs (p_H 1·2–10·0) were employed. 20 cc of buffer solution with a similar amount of distilled water were put into the jars, and 5 cc of buffer solution with 5 cc of 40% glucose solution into the tubes. The influence of McIlvaine's buffers was first determined. Experiments were run for three hours with fresh and for half an hour with treated membranes. The results of typical experiments are shown graphically in figs. 3 and 4.

Glucose penetrates the treated membranes very quickly. Taking the figures at their face value (allowing for the shorter duration of the experiments with treated membranes), glucose at p_H 2·2 passes from tube A some 230 times faster after the lipin has been removed from the membrane. Somewhat similar curves were obtained for both series of experiments and for both " A " and " B " membranes. A comparatively high speed of penetration at the acid end of the range is followed by a sharp drop and then by a sudden rise and by a final drop. A comparison between the curves for fresh and treated membranes reveals that the lowest point on the curve on the acid side does not occur at the same p_H. In the fresh membranes it lies at about p_H 5·0 for both " A " and " B " membranes, while in treated membranes at p_H 3·6 in each case. As already recorded (Yonge, 1932), the *iso*-electric points of the cuticle and chitin lie at about p_H 5·1 and 3·5 respectively. This provides the probable explanation of this difference between the effect of p_H on the permeability of the fresh and treated membranes. The point of especial interest is that in the fresh membranes it is the *iso*-electric point of the *cuticle* which influences

permeability whereas in the treated membranes this has no longer any influence but the *iso*-electric point of the *chitin* now exerts its effect.

If hydrogen ion concentration alone influenced the permeability of the membranes, a continual rise on either side of the *iso*-electric point would be expected. This is actually so on the acid, but not on the alkaline, side where, after a peak at about p_H 6·0, there is always a drop. Clearly other factors are at work, probably the action of ions other than hydrogen.

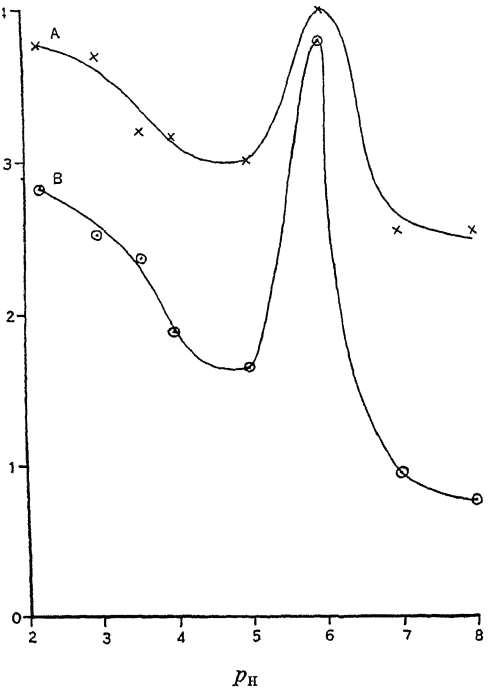

FIG. 3—Graph showing influence of McIlvaine's buffer solutions on passage of glucose through fresh membranes, " A " and " B ". Ordinates represent milligrams of glucose penetrated in 3 hours.

This is discussed after the results of the experiments with Clark and Lubs's buffer solutions have been described. These experiments were run for two hours with fresh and for half an hour with treated membranes, results being shown in figs. 5 and 6.

When a comparison is made between the results for the fresh membranes with the two sets of buffers (compare figs. 3 and 5) certain obvious differences are seen. With Clark and Lubs's buffers the lowest point on the acid side lies at p_H 4·0 instead of at 5·0. Although there is a peak at p_H6·0 (KHPhthalate-NaOH) the permeability to glucose at p_H 6·0 (KH_2PO_4-NaOH) is very much less. There is then a gradual rise in

permeability in the same series up to p_H 8·0 but again the figures for p_H 8·0 (H_3BO_3, KCl-NaOH) are much lower. Finally there is a slight fall at p_H 9·0 followed by a considerable rise at p_H 10·0. The permeability of the fresh membrane is certainly being influenced by some factor other than hydrogen ion concentration, and this appears to be the actual concentration of sodium ions in any given buffer solution. With the first two buffer series (KCl-HCl and KHPhthalate-HCl), where no sodium ion is present, permeability is essentially the same as with McIlvaine's

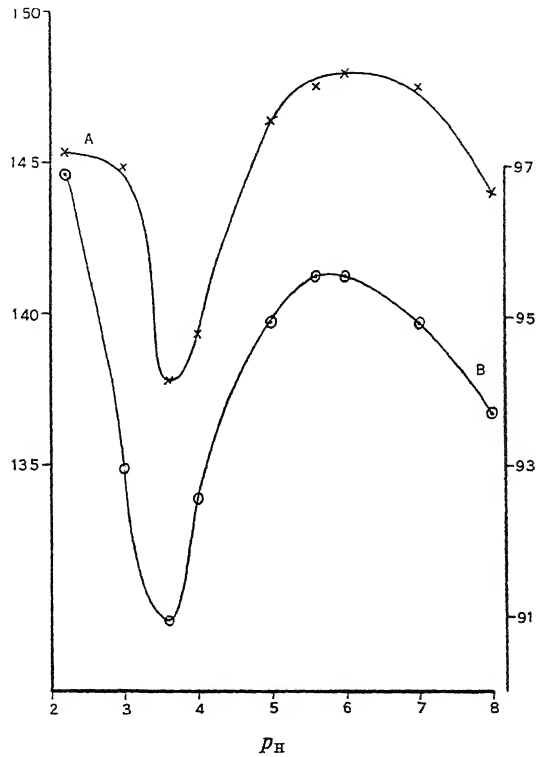

FIG. 4—Graph similar to that in fig. 3 showing passage through treated membranes, experiments for ½ hour.

buffers, but in the last three where the variable constituent in each is the amount of sodium ion (the quantity of the other constituents remaining constant in each series) there is a rise from p_H 4·0 to 6·0, from 6·0 to 8·0, and from 8·0 to 10·0. Where the hydrogen ion concentration is the same in different buffers, as at p_H 6·0 and 8·0, higher permeability is associated with higher concentration of sodium ions. The fact that permeability is greater at p_H 5·0 than at p_H 4·0—unlike the results with McIlvaine's buffers—may thus be attributed to the different concentrations of sodium ion.

If the varying concentrations of the cation Na are responsible for the peculiar results obtained, it might be expected that the increasing concentration, from p_H 2·2 to 8·0, of sodium phosphate in McIlvaine's buffers would have a similar effect. This did not occur when experiments were conducted beginning at the acid end of the range, but did occur in certain cases when experiments were started at the alkaline end indicating

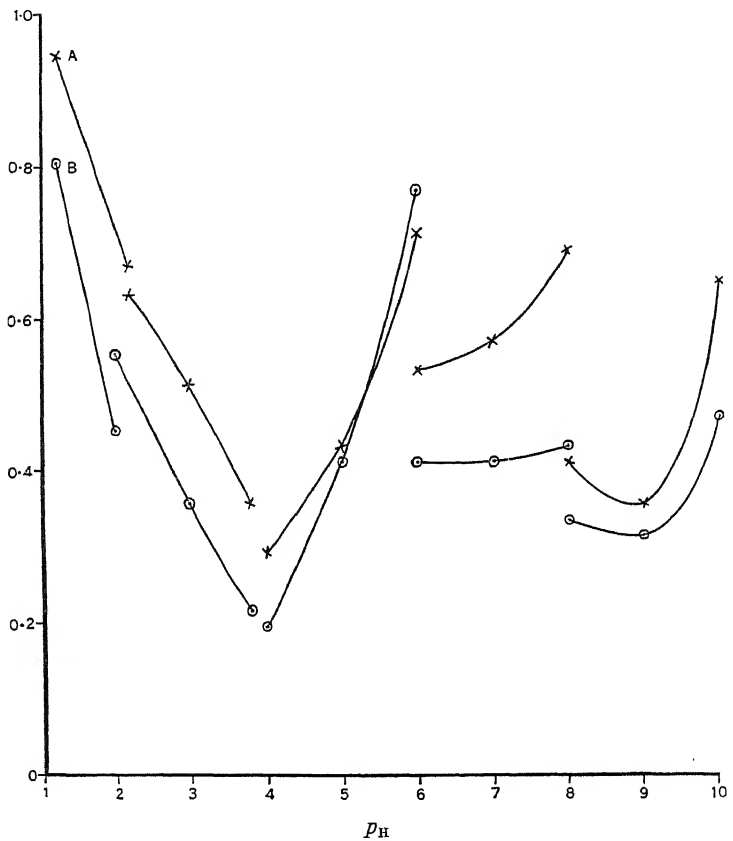

FIG. 5—Graph showing influence of Clark and Lubs's buffer solutions on passage of glucose through fresh membranes, " A " and " B ". Ordinates represent milligrams of glucose penetrated in 2 hours.

that initial exposure to a strong concentration of a sodium salt *may* affect the apparently unstable surface of the cuticle.

In the treated membranes, as shown in fig. 6, the effect of hydrogen ion concentration is predominant, and, though figures for the different buffers do not entirely agree, curves of the same general character as those for McIlvaine's buffers, fig. 4, can be drawn through the various points. The lowest point on the acid side is nearer p_H 3·0, and the peak

of the curve lies at about p_H 7·0, but these differences are possibly the result of differences between the constituents of the buffer solutions, Vonk (1931) having shown that the swelling of colloids is influenced by specific ions as well as by hydrogen ion concentration. Permeability in the treated membranes is possibly affected by the influence of ions on the hydration of the membrane.

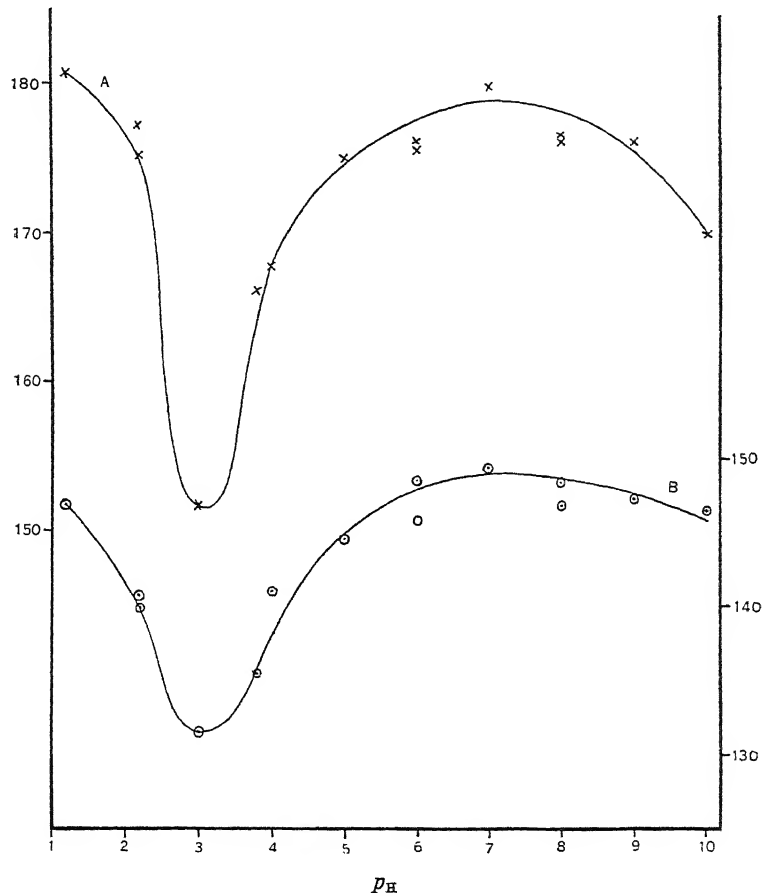

FIG. 6—Graph similar to that in fig. 5, showing passage through treated membranes, experiments for ½ hour.

XI—THE EFFECT OF TEMPERATURE

The effect of temperature on the passage of a fat solvent through both types of membrane was examined, formic acid being used. Experiments, details of which are given in Table VII, were carried out at 20°, 30°, 40°, and 50° C, and finally again at 20° C.

If the passage of formic acid through the fresh membranes had been assisted in any way by a chemical reaction a considerable *progressive* increase in the rate of passage through the membranes with increasing temperature should have been revealed. The results recorded in Table VII, and the graphs in fig. 7 which are straight lines, indicate that passage is probably by diffusion.

TABLE VII—EFFECT OF TEMPERATURE ON PASSAGE OF FORMIC ACID THROUGH FRESH AND TREATED MEMBRANES

0·1N acid used; experiments for 1 hour; other details as in Table I

Tube	% passed through membrane					% increase per degree rise in temperature, value at 20° C taken as 100		
	20° C	30° C	40° C	50° C	20° C	20–30° C	20–40° C	20–50° C
				Fresh membranes				
A_2	3·5	6·2	9·5	13·4	5·5	7·9	8·6	9·4
A_{14}	6·2	13·0	19·1	24·5	7·0	10·8	10·3	9·7
					Average =	9·35	9·45	9·55
B_{21}	8·7	14·2	19·0	24·7	11·0	6·3	5·8	6·1
B_{22}	10·0	15·0	19·5	24·7	12·2	5·0	4·8	4·9
					Average =	5·65	5·3	5·5
				Treated membranes				
A_4	18·0	21·4	24·5	29·5	18·0	1·9	1·8	2·1
A_6	21·0	24·9	28·5	32·7	21·0	1·8	1·8	1·9
B_3	16·0	19·2	21·5	24·5	16·5	2·0	1·7	1·8
B_{13}	20·7	23·7	27·7	30·2	20·9	1·5	1·7	1·5
					Average =	1·8	1·75	1·8

Additional evidence that the lipin assists the passage of fat solvents is provided by the much higher temperature coefficient for their passage through fresh membranes. Moreover in fresh, though not in treated, membranes the second experiment at 20° C always showed increased permeability, *i.e.*, some of the lipin has been removed, or in some way altered. Finally in fresh membranes alone the percentage increase per degree rise in temperature is very much higher in "A" than in "B" membranes. This agrees with the conclusion arrived at in § IX that the two interfaces, cuticle-chitin and cuticle-water, are in some way different.

XII—Passage of Substances through Membranes Suspended in Air

All experiments hitherto described have dealt with the passage of solutes from one side of the membranes into water or some other solvent on the other. A quantity of important data, summarized in Table VIII, was obtained by suspending tubes in air and observing the passage of liquids through the membranes.

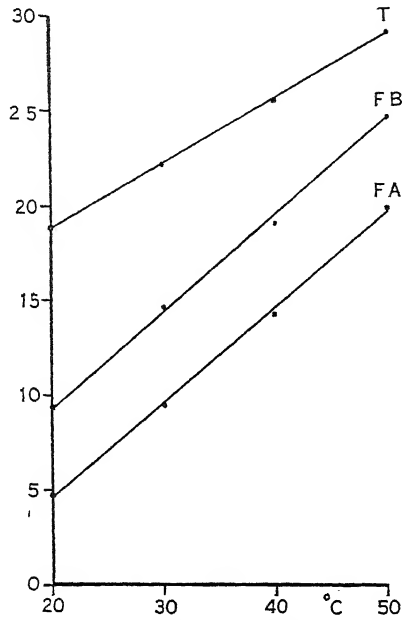

Fig. 7—Graph showing effect of temperature on rate of passage of 0·1N formic acid through fresh membranes (" A " and " B ") and treated membranes. Ordinates indicate percentage passed during 1 hour. Data given in Table VII.

Table VIII—Passage of Liquids through Fresh Membranes Suspended in Air

Liquid	Passage	
	" A "	" B "
Distilled water	—	—
N formic, acetic, propionic, or butyric acids	—	+ freely
N HCl, HNO_3, or H_2SO_4	—	—
N NaOH or KOH	+ immediately	+ immediately
N NH_4OH	—	+ freely
Neutral salts	—	—
Absolute alcohol	—	—
Acetone	—	—

Treated membranes allowed aqueous solution of all kinds to pass immediately but did not permit the passage of either alcohol or acetone. After the effective removal of the cuticle the treated membrane, *i.e.*, chitin, apparently acts as a simple diffusion membrane allowing all liquids to pass freely with the exception of those, such as alcohol or acetone, which dehydrate it. With these the under side of the membrane becomes hard and dry and stretches very tightly.

The fresh membranes, on the other hand, when suspended in air are impermeable to many substances. Water, strong mineral acids in normal concentration, and neutral salts, do not pass through in either direction. Fatty acids and ammonia pass readily through " B " membranes, *i.e.*, from the cuticle to the chitin side, but *never* in the reverse direction. Alcohol and acetone never pass in either direction and for the same reason that they fail to pass through treated membranes. The only substances which penetrate " A " as well as " B " membranes under these conditions are the strong alkalies, sodium and potassium hydroxides (similar results were obtained with saturated solutions of barium and calcium hydroxides but over longer spaces of time). This presumably is due to the direct action of these alkalies on the substance of the cuticle, a reaction which was utilized in the preparation of treated membranes.

The chitin itself is probably not affected by the treatment involved in the preparation of treated membranes. This was indicated by attaching fresh membranes with the cuticle side undermost to tubes and then gradually scraping away the cuticle with fine sand paper. When the cuticle had been removed water, previously retained in the tubes, began to drip through in that area. The tube was then emptied, dried, and refilled with absolute alcohol. This quickly dehydrated the membrane and further passage of liquid was prevented. It is thus possible to prepare treated membranes by the simple removal, by abrasion, of the cuticle. The chitin, of which the membrane is then exclusively formed, behaves exactly as do treated membranes. This method cannot be recommended in practice because it is impossible to remove the cuticle over a wide area while as soon as it is removed the relatively very much softer chitin perforates very readily.

XIII—Possible Passage of Fat Solvents through Fresh Membranes when the Same Concentration is Present on Both Sides

The unidirectional passage of fatty acids and ammonia through fresh membranes suspended in air led to the carrying out of extensive series of experiments with a variety of acids and alkalies with the same con-

centration present on both sides. Measurements of volumes and titrations indicated that under these conditions there was a passage with fatty acids and ammonia exclusively and always in the one direction, cuticle to chitin. The maximum changes recorded, after experiments of 46 hours' duration, were an increase within tubes with "A" membranes of 4·1% and a decrease with "B" membranes of 4·5%. The presence of cyanides did not affect these results.

Later potentiometer readings were made using both calomel and quinhydrone electrodes, but no significant difference in potential, or in p_H with the acids, between the two sides of the membrane could be found. Nevertheless the results obtained by titrations would be of such interest, if confirmed, that they appear worthy of record.

XIV—Discussion

The permeability of the uncalcified integument of the Decapod Crustacea, as exemplified by the material used, is *controlled absolutely* by the properties of the cuticle secreted by the tegumental glands. The presence of this converts what would otherwise be a freely permeable membrane of chitin into one with variable degrees of permeability. This appears to be due primarily to the presence within the cuticle of a lipin, probably lecithin or some closely allied compound, which has previously been demonstrated histochemically (Yonge, 1932). This lipin renders the cuticle readily permeable to fat solvents but only very slightly permeable to other substances whether electrolytes or non-electrolytes. Nevertheless these penetrate rapidly enough for it to be demonstrated that monovalent cations pass through significantly more quickly than divalent cations. The passage of non-electrolytes, such as glucose, is influenced by hydrogen ion concentration, permeability being notably lowered about the *iso*-electric point of the cuticle, but this is masked to some extent by the action of specific ions. Finally the failure of acetone and alcohol to penetrate when the membrane is suspended in air indicates that permeability is dependent on water content, which agrees with the experiments on the effect of hydrogen ion concentration, the water content of a colloid being lowest at the *iso*-electric point.

A most interesting point is the difference which exists between the nature of the cuticle-chitin and the cuticle-water interfaces, and which results in fat solvents passing through fresh membranes exclusively in the direction cuticle to chitin when no fluid is present on the other side. On the other hand divalent cations penetrate with considerably greater

speed in the opposite direction (although in both cases passage is greatly retarded in comparison with that of monovalent cations).

Chitin itself is a simple diffusion membrane allowing all fluids, other than those which cause dehydration, to pass freely. Electrolytes pass through strictly in accordance with the mobilities of the ions. The permeability of the chitin is influenced by p_H, but it is now the *iso*-electric point of the chitin and not of the cuticle which determines the position of minimum permeability. The action of other ions is less well-marked than when the cuticle is present, the passage of divalent cations not being retarded in relation to that of monovalent cations, effects on the membrane being apparently confined to influencing hydration.

Previous work may now be considered in the light of these results. Wigglesworth (1933, *b*) has recently shown that, unlike the Decapod Crustacea, the dermal glands in the Insecta, as exemplified by those of *Rhodnius prolixus*, play no part in the formation of the integument, being concerned exclusively with the secretion of the moulting fluid. But he has been able to confirm the findings of Kühnelt (1928) that the surface layer or epicuticle has many properties in common with the cutin or suberin of plants, being, in Wigglesworth's words, "perhaps a complex mixture of fatty or waxy substances". It is formed, possibly by the oenocytes, which Wigglesworth thinks may synthesize the non-chitinous constituents of the integument, before the underlying endocuticle, which is composed largely of chitin, but into which it may penetrate (there is not that complete division between the two layers which occurs in the Crustacea as a result of their distinct origin). It is clear, therefore, that there are important distinctions between the integument of the Insecta and the Crustacea, but at the same time the surface layers in both groups have peculiar properties associated in part with the presence in them of substances of a fatty nature. The work of Petrunkevitch (1900), of Sanford (1918), and of Abbott (1926) shows that the epicuticle of Insecta must have properties in common with that of the cuticle of Crustacea, because fatty acids will readily pass through the integument of the crop in the cockroach. The great permeability of the peritrophic membrane in *Glossina* (Wigglesworth, 1929) may be due to the absence of any epicuticle; similarly that of the chitin lining the midgut of Isopoda is almost certainly explicable by the absence here of the tegumental glands which alone can form the cuticle of Crustacea.

It has been usual to regard chitin as the most important constituent in the endoskeleton of a variety of animals and as being little more than a semipermeable membrane even when not impregnated with other substances. The results recorded in this paper compel the revision of

these opinions. Chitin lacks both rigidity and semi-permeability. Rigidity is supplied by impregnation with calcium salts or with scleroproteins (in Decapod Crustacea and Insecta respectively), Campbell (1929) and Tauber (1934) having emphasized the fact that the denser parts of the insect integument, usually referred to as being "heavily chitinized", actually contain less chitin than do the thinner, transparent regions. Permeability, where it is not influenced by such impregnation or before it is so influenced (*i.e.*, during the formation of the new integument at ecdysis), is profoundly modified by the cuticle in the Crustacea, and probably by the epicuticle in the Insecta. Neither of these consists of chitin. Chitin forms an ideal *matrix* for an endoskeleton, being capable of impregnation where protection or rigidity is needed and, when left unimpregnated, providing the flexible membrane essential at joints.

We are now in a position to consider the significance of the cuticle, the importance of which is sufficiently indicated by the ubiquity and efficiency of the tegumental glands which produce it. Wigglesworth (1933, *b*) considers that the function of the epicuticle in the Insecta is to prevent the dissolution of the new integument when the underlying layers of the old integument are being dissolved before ecdysis by the exuvial fluid. This is clearly one of the functions—probably the primary function—of the cuticle in the Crustacea. In *Homarus*, as previously shown (Yonge, 1932), the underlying surface of the old chitin is dissolved by the action of wandering cells (an interesting difference from the mechanism in the Insecta) while it was further shown that chitinase (obtained from the stomach juices of *Helix*) does not attack the cuticle. It is clear that the old integument could not be freed from the epithelium and so the new integument be able to form beneath if the latter were not protected by some non-chitinous layer.

But although this almost certainly represents the primary function of the cuticle this has other, and hardly less important, functions. The chitin could be protected from dissolution equally well by a substance which did not possess lipin and did not control permeability. The presence of the cuticle has solved one problem presented by an endoskeleton which must be cast from time to time, it has also solved another—the free permeability of unimpregnated chitin. There is no obvious disadvantage in the possession of a freely permeable integument so long as the underlying epithelium can regulate the passage of solutes into and out of the body. This is performed by the epithelium of the gills in shore-living Decapoda such as *Carcinus maenas* (Schlieper, 1929; Margaria, 1931; Bateman, 1933). Conditions may possibly be different while the epithelium is engaged in actively secreting the chitinous integument. It is

possible that under these conditions the epithelial cells lose their powers of regulation. It is certainly true that the aquatic Crustacea take in water freely immediately after the old integument is shed and before the new one has been hardened by the impregnation of calcium salts. The presence of the cuticle would in no way affect the passage of water but it would restrict the passage of solutes (other than fat solvents or strong alkalies which are not present under natural conditions). It should be realized that when the old integument is cast, although the cuticle is fully formed, the underlying chitin is still being produced by the epithelial cells (Vitzou, 1882; Yonge, 1932).

The cuticle has a third function easier to demonstrate. Where the chitin is never impregnated with calcium salts, for instance in the foregut and hindgut and over the gills in the Decapoda, and over the entire surface in the lower Crustacea, it serves as a protective layer. It is much harder than the chitin as was proved by the attempts to remove it by abrasion. In the foregut of the Decapoda, which is essentially a crushing gizzard, the integument is exposed to continual abrasion in life. It is frequently filled with jagged pieces of calcareous matter. At the same time the integument must remain supple or the gastric mill could not function. The unprotected chitin would certainly be perforated. Herein lies the explanation for the immense aggregations of tegumental glands in the labrum and oesophagus (Yonge, 1932) which secrete the abnormally thick cuticle present in these regions and in the stomach. Tegumental glands are unusually abundant in the hindgut, and also in the gills (Allen, 1893).

The cuticle is thus a most important substance. It protects the new integument from dissolution when the old one is being removed, its limited permeability may be of vital assistance after the old integument has been cast but before the epithelium has completed the secretion of the new chitin, and it certainly provides a protective layer essential when the chitin is not impregnated. In addition it has been used for a variety of subsidiary purposes, namely for the formation in the Decapoda of the outer, cementing layer round the eggs (Yonge, 1935), and of the cement which secures the statoliths to the sensory setae (Lang and Yonge, 1935), and in certain Amphipoda for the formation of the material which forms the nest (unpublished work).

There is a striking analogy between the permeability of the fresh membranes and those of the living cell membrane. So far as can be ascertained this is the first non-living membrane of *animal origin* revealed to have such properties, and certain facts that have emerged from its study appear worthy of note.

The fresh membranes possess in the cuticle a lipoid substance, the influence on which of monovalent and divalent ions and also of hydrogen ion concentration can easily be studied. Of very particular interest is the difference, which undoubtedly exists, between the nature of the two interfaces, cuticle-chitin and cuticle-water, and which is apparently responsible for the unidirectional passage of fatty acids and ammonia through membranes suspended in air, and *possibly* when the same concentrations of these substances are present on both sides. An elucidation of the underlying mechanism responsible for the differences between the two surfaces of the cuticle would be of great interest.

The greater part of this work was carried out at the Plymouth Laboratory when the author was a member of the staff. Certain additional experiments were carried out with assistance from the Colston Research Fund of the University of Bristol. The author is particularly indebted to Dr. E. J. Allen, C.B.E., F.R.S., for his interest and help and to Dr. W. R. G. Atkins, F.R.S., for advice and criticism.

XV—Summary

The permeability of the integument lining the foregut of *Homarus* is profoundly influenced by the bounding cuticle which contains a lipin, probably lecithin or some closely allied substance.

The cuticle can be effectively removed by appropriate treatment. The permeability of such "treated membranes", which consist effectively of chitin alone, was compared to that of "fresh" membranes with intact cuticle.

Fatty acids and hydrochloric acid penetrate fresh membranes in the order, formic > propionic > butyric > acetic > hydrochloric, and treated membranes in the order, hydrochloric > formic > acetic > propionic > butyric, *i.e.*, in the order of their degree of dissociation.

The strong mineral acids penetrate both types of membrane in the same order, nitric > hydrochloric > sulphuric.

Alkalies penetrate fresh membranes in the order, $NH_4OH > KOH > NaOH > LiOH > Ba(OH)_2$, and treated membranes in the order, $KOH > NaOH > Ba(OH)_2 > LiOH > NH_4OH$. The passage of barium hydroxide is inhibited in comparison with that of potassium hydroxide through fresh membranes, almost twice as much in the direction cuticle to chitin as in the reverse direction.

Molar solutions of chlorides penetrate fresh membranes in the order, $HCl > NH_4CL > KCl > NaCl > LiCl > CaCl_2 > MgCl_2 > CdCl_2$. In

treated membranes the positions of ammonium chloride and potassium chloride are reversed. The passage of the chlorides with divalent cations is greatly inhibited by the cuticle, again distinctly more in the direction cuticle to chitin.

Glucose penetrates fresh membranes extremely slowly but treated membranes rapidly. In fresh membranes permeability is notably lowered at the *iso*-electric point of the cuticle and in treated membranes at that of the chitin. Permeability through both types of membrane is influenced also by the action of ions other than hydrogen.

Experiments on the effect of temperature on the passage of formic acid in aqueous solution indicates that the greater speed of passage of fat solvents through fresh membranes may be due to assistance in the rate of diffusion.

When suspended in air fresh membranes are permeable only to fat solvents, in the direction cuticle to chitin only, and to strong alkalies which attack the substance of the cuticle. Treated membranes act as simple diffusion membranes, only substances which dehydrate them fail to pass through.

Chitin itself is an admirable skeletal matrix possessing the flexibility essential at joints. It lacks the rigidity and limited permeability with which it has been credited. The former is supplied by impregnation with scleroproteins or calcium salts, the latter by the cuticle which converts the freely permeable chitinous membrane into one with limited and variable degrees of permeability.

The primary function of the cuticle is probably the protection of the new chitin when the old chitin is being dissolved in the early stages of ecdysis. The limited permeability of the cuticle may be of great importance at ecdysis when the regulative powers of the epithelium are possibly in abeyance during the secretion of chitin.

The harder cuticle protects the thicker, but much softer, chitin from abrasion in regions where this is not impregnated.

There are striking analogies between the permeability of the cuticle and that of the living cell membrane.

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Carbonic Anhydrase and Photosynthesis

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During the past few years, the properties of a new enzyme, carbonic anhydrase, have been studied. This work is described in detail by Roughton (1934). Henriques (1928) had pointed out that the rate of evolution of CO_2 from the lungs was much more rapid than the evolution of CO_2 from a carbonic acid solution and concluded that: (*a*) there must be a catalyst present, or (*b*) the CO_2 was carried in some form other than bicarbonate. Brinkman, Margaria, and Roughton (1933) confirmed the view of Faurholt (1924) " that in the range p_H 0–8 the equation

$$\frac{d\,[CO_2]}{dt} = k\,[H_2CO_3] - k'\,[CO_2],$$

corresponding to the mechanism 1 mol CO_2 + 1 mol $H_2O \rightleftarrows$ 1 mol H_2CO_3, holds good ".

The values of k and k' given by Brinkman, Margaria, and Roughton are compared with those of Faurholt in Table I.

TABLE I

	0° C		18° C	
	Faurholt	Brinkman, Margaria, and Roughton	Faurholt	Brinkman, Margaria, and Roughton
k	2·67	1·70	16·4	11·1 (average)
k'	0·0030	0·0026	0·025	0·024 ,,

Roughton (1934) pointed out that the most obvious function of carbonic anhydrase is to accelerate the formation of carbon dioxide from bicarbonate in the lung blood-vessels of air breathing animals. It is estimated that this reaction must be accelerated 200 times if the lung mechanism is to be efficient. A sufficient concentration of enzyme has been found in human blood to increase the rate of reaction about 2000 times at 15° C and about 600 times at 37° C.

No carbonic anhydrase was found by Roughton in green plant tissue. Preliminary experiments done in this laboratory, using the exact technique of Roughton, have failed to demonstrate any acceleration by extracts of

leaf cells ground with sand. Since leaves of land plants are so constructed that most of the cells are exposed to air spaces, the CO_2 comes off directly from each cell and there is no point of extremely rapid evolution of gas as found in lung tissue. Probably no enzyme would ever be required to dehydrate the H_2CO_3 of respiration in plants.

Photosynthesis, on the other hand, may proceed at a rate 10 to 20 times as high as respiration. Furthermore, CO_2 is entering in solution and must be hydrated instead of dehydrated if used as H_2CO_3. The constant for this reaction at 18° C (Table I) is about 1/500 of that for dehydration. The rate at which H_2CO_3 will be formed from CO_2 when no H_2CO_3 is consumed in photosynthesis is given by the expression

$$\frac{d\,[H_2CO_3]}{dt} = k'\,[CO_2] - k\,[H_2CO_3].$$

If it is assumed that the concentration of H_2CO_3 is held at zero by immediate use in photosynthesis, the rate reaches a maximum, and at 18° C, it is

$$\frac{d\,[H_2CO_3]}{dt} = 0{\cdot}024\,[CO_2],$$

in the absence of a catalyst. This is the maximum rate of photosynthesis if H_2CO_3 is the reactant.

By making certain assumptions, the maximum possible rates of formation of H_2CO_3 in leaves can be compared with observed rates of photosynthesis. First, assume that the tissue is saturated with CO_2 from an atmosphere of known CO_2 concentration. Second, assume that the entire weight of tissue is water capable of dissolving CO_2. The latter assumption is at least 10% out since leaves are usually only 90% water. The former is likely to give results too high during active photosynthesis. But both of these assumptions will be used in order to get the *maximum possible rate* of photosynthesis, if limited by the uncatalysed hydration of CO_2.

For submerged plants, the best available data are given by van den Honert (1930) who used *Hormidium* filaments under a film of water estimated to be 8 μ thick. The CO_2 entered this film directly from the air. The average unit quantity of *Hormidium* was about 45 metres long and 8 μ in diameter, making a volume of approximately 2·25 c mm. Under optimum conditions of temperature and light and CO_2 this unit assimilated 100 c mm CO_2 per hour.

The solubility of CO_2 in water at 20° C and 760 mm pressure is 0·1688 gm per 100 cc, or 85 cc of CO_2 per 100 cc of H_2O. With 0·01 vol. %

CO_2 in the air, the maximum solubility is reduced to 0·0085 cc per 100 cc. Therefore, the 2·25 c mm of *Hormidium* tissue could contain 0·000191 c mm CO_2 at 20° and 0·01 vol. %.

The maximum rate of uncatalysed hydration at 18° C is 0·024 × c mm CO_2 in solution per sec or 86·4 × c mm CO_2 in solution per hour. For the unit tissue of *Hormidium* there would be 86·4 × 0·000191, or 0·0165 c mm CO_2 hydrated per hour.

But at 0·01 vol. % CO_2, van den Honert found that the unit of *Hormidium* assimilated 40 c mm CO_2 per hour. Therefore, the observed rate of photosynthesis is more than 2400 times the maximum rate of uncatalysed hydration of CO_2.

The presence of a film of water over the algae affects the absolute value of this calculation, but in any case, the rate of photosynthesis can be shown to be far greater than the maximum rate of uncatalysed hydration of CO_2.

Leaves furnish more reliable data for calculations since the total volume in which hydration can take place must be less than the weight of the leaf tissue.

Boysen Jensen (1932) found an assimilation in normal air of 8 cc CO_2 per 100 sq cm leaf surface of *Sinapis*. The weight of the leaf is not given, but it is a thin-leaf type. 100 sq cm of thin leaves (including ribs) weigh from 1·5 to 1·7 gm. Therefore, 1·5 cc of water available for solution of CO_2 can be set as a maximum in 100 sq cm of *Sinapis*. Using the same data as for the previous calculation, we find that the maximum CO_2 soluble in the leaf in air is 1·5 × 0·85 × 0·0003 = 0·00038 cc. Therefore, the maximum rate of uncatalysed hydration of CO_2 is 86·4 × 0·00038 = 0·033 cc/100 cm^2/hour. The observed rate of 8 cc per hour is 242 times as much.

A similar calculation made from the data of Brown and Morris (1893) and recalculated by Blackman and Matthaei (1905) for *Helianthus* leaves gives a ratio of rate found to uncatalysed rate of 182. Probably this value is very low since Brown and Morris used the method of increase in dry weight and made no correction for translocation.

In this laboratory, Jeffrey (unpublished data) has found that the rate of assimilation by *Pelargonium* may reach 13·4 cc/100 sq cm/hour in 0·1% CO_2. *Pelargonium* has a relatively thick leaf with a mass of about 2·5 gm per 100 sq cm when heavy ribs are excluded. Still, the rate found is 74 times the calculated.

The calculations are summarized in Table II.

The observed rate is always much greater than the calculated maximum hydration rate if no catalyst is present. Preliminary experiments have

TABLE II

Author	Plant used	Rate of assimilation per unit of tissue	Volume of unit	Rate of assimilation per cc of tissue per hour cc	External CO_2 conc. vol %	Maximum solubility of CO_2 in 1 cc of tissue cc	Maximum uncatalysed rate of CO_2 hydration per hour cc	Ratio of rate found to uncatalysed rate
van den Honert....	*Hormidium* ..	40 c mm/unit/hr	2·25 c mm	17·8	0·01	0·000085	0·0073	2438
Boysen Jensen	*Sinapis*	8 cc/100 sq cm/hr	1·5 cc	5·33	0·03	0·000255	0·022	242
Brown and Morris	*Helianthus*	8 cc/100 sq cm/hr	2·0 cc	4·00	0·03	0·000255	0·022	182
Jeffrey	*Pelargonium* ..	13·4 cc/100 sq cm/hr	2·5 cc	5·36	0·10	0·00085	0·073	73

failed to demonstrate the presence of the catalyst in leaf tissue. If there is no carbonic anhydrase in leaf tissue the theory must be abandoned that H_2CO_3 becomes associated with chlorophyll as a magnesium bicarbonate (Stoll, 1932). In its stead, a mechanism can be assumed by which CO_2 is associated directly with an activated chlorophyll molecule. The series of equations suggested by Dixon and Ball (quoted by Spoehr, 1926) and Adams (1926) are good examples of the use of CO_2 rather than H_2CO_3.

It has been shown (Gaffron, 1935; Roelofsen, 1935) that gaseous hydrogen can be used by bacteria (*Athiorhodaceae* and *Thiorhodaceae*) for carbon dioxide assimilation. In the series of reactions given by Stoll (1932) the active hydrogen could reduce CO_2 rather than H_2CO_3, the chlorophyll deriving the hydrogen from its water of hydration. The general photosynthetic equation of van Niel (1931) would then also hold good for green plants:

$$CO_2 + 2H_2A \rightarrow CH_2O + H_2O + 2A,$$

where A is a chlorophyll nucleus.

A further search must be made for carbonic anhydrase in green plant tissues before a more serious consideration can be given to this enzyme in determining rate and mechanism of photosynthesis.

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Summary

It has been found that the observed rate of photosynthesis by green plants is far greater than the rate of uncatalysed hydration of CO_2. The constants for the latter reaction are taken from extensive work done in studies on carbonic anhydrase present in animal tissues.

Preliminary experiments with ground leaf tissues have failed to demonstrate the presence of a catalyst for CO_2 hydration. These findings throw doubt on the view that CO_2 must become hydrated to H_2CO_3 before reacting with chlorophyll.

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The Mechanism of the Bactericidal Action of Radioactive Radiations

I—Theoretical

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1—Introduction

Much has been published upon disinfection by X-rays and the alpha, beta, and gamma radiations from radioactive substances, *see* for example, reviews by Packard (1931) and Lacassagne and Holweck (1934). The mechanism of disinfection, however, remains obscure. Theories have been proposed, but little attempt seems to have been made to analyse the implications of the various hypotheses and point by point to devise experiments to confirm or disprove them. Moreover, some writers have ignored the fact that the physical processes accompanying the passage of the various radiations through matter are fairly completely understood, and have suggested mechanisms incompatible with this knowledge.

During the present research the authors had in mind three alternative hypotheses of the mechanism of disinfection by radiation, namely:—

(1) secondary poisoning of the cell by chemical substances liberated in it by the radiation;
(2) destruction of essential constituents of the cellular protoplasm by the radiation;
(3) the " target hypothesis ".

The experiments so far performed have been designed to decide between these mechanisms. As is to be expected when the models are too crude, there are some experiments which argue against each, indicating the necessity for modification and amplification of the working hypotheses.

2—Physical Phenomena Accompanying the Passage of Radiations through Matter

Of the four types of radiation considered, alpha, beta, gamma, and X-rays, there is no fundamental difference to be expected in the biological effects of the three last mentioned. The absorption of an X-ray or gamma ray quantum of the energy here considered takes place either by photoelectric effect or by Compton effect. In the former the whole energy is lost (principally to an electron) in a single process; in the latter the photon is scattered with loss of energy and may experience the process more than once before finally disappearing by photoelectric absorption. The photon thus disappears often after only one atomic encounter, and always after a small number, and its primary action is for the present purposes negligible, the biological effects being due to the secondary electrons produced. These electrons will have a variety of energies with an upper limit fixed by the quantum energy of the primary radiation. On any theory of the biological action of radiation there is therefore bound to be quantitative agreement between the effects of X-rays (or gamma rays), and of cathode rays (or natural beta rays) having energies similar to those of the secondary electrons produced by the X- or gamma rays, the rates of disinfection being equal for equal rates of energy absorption per unit volume.

The X-ray dose required to kill a bacterium has sometimes been expressed in terms of the mean number of quanta required to be absorbed in the organism to produce death; it is evident that, except for very soft radiation, this mode of expression is not of any particular significance, and if the number found happens to be one quantum per bacterium, such a result can only be considered accidental.

Attention can be confined therefore to alpha and beta particles. Most of the physical data which exist refer to the passage of these particles through gases. The energy absorption and the production of ionization in a given mass of material is, however, independent of the physical state and is only slightly dependent upon the state of chemical combination, and the phenomena in the solid and liquid phase can thus usually be inferred from experiments in the gaseous phase. When an alpha or beta particle passes through matter it undergoes numerous collisions with the molecules in which energy is transferred varying in amount from a few electron volts to several hundred electron volts. The less energetic collisions result in excitation, the more energetic in ionization. As a result of the latter process (primary ionization) electrons (or delta rays) are liberated, a proportion of these have sufficient kinetic energy to enable them to produce further excitation or (secondary) ionization. The production of delta rays is an accompaniment of the passage of both alpha and beta rays through matter, and secondary ionization in both cases constitutes about two-thirds of the total ionization. It is evident that as far as the production of ionization is concerned, the two types of particle differ only in the fact that the alpha particles make many more primary ionizing collisions in a given path than do the beta particles.

The excited or ionized molecules being in a state of energy higher than the normal are more active chemically; complex molecules are likely to suffer decomposition while simple molecules are likely to combine. Thus H_2O, H_2S, NH_3, and similar substances are decomposed into their constituent elements, while hydrogen and bromine are caused to combine with production of HBr, ozone is produced from oxygen, and so on (Lind, 1928). In most reactions of this type the number of molecules undergoing chemical change is of the same order as the number of ions formed.

The passage of radiations through an organism thus gives rise to ions, which chemically are foreign to the organism and which initially are electrically charged and are endowed with energies in excess of kinetic theory energies. The biological effects of the radiation must be ascribed to one or more of these three characteristics distinguishing the ions from the original molecules. It is unlikely that the electrical charge is significant, since separation of charge already occurs to a much greater extent whenever inorganic salts are present in solution. One is limited then to the chemical change and the energy increase. Both of these have from time to time been suggested as the cause of the biological effects of radiation. Consider first the energy increase explanation. At collision

between ions of opposite sign the energy of ionization may be converted into kinetic energy, and similarly at collisions between excited and normal molecules. As a result of these collisions of the second kind there will be molecules in the medium in the neighbourhood of the path of the ionizing particle having energies of the order of some electron volts. On the kinetic theory which associates temperature and mean molecular energy, one may thus loosely consider the passage of the ionizing particle to have produced a localized region of high temperature. This point of view has been put forward by Dessauer (1923) and accepted by various writers who have predicted temperature rises of between 10^{20} and a few hundred degrees. It is very doubtful however if this notion of local heating is worth retaining. Strictly, temperature only has meaning when there is a Maxwellian distribution of velocities among the molecules. Immediately after the passage of the ionizing particle such a distribution does not exist; hence the wide variation of estimates which have been made of the temperature rise. By the time a Maxwellian distribution has been achieved, the energy has become distributed over a larger volume and the temperature rise due to a single ionizing particle is insignificant. In view of these considerations it is not to be expected that there should be any close correlation between the biological effects of radiation and the biological changes accompanying a rise of temperature; it is preferable not to consider the radiation from the macroscopic point of view as producing a local heating, but to regard the phenomenon from a molecular standpoint. In these terms one may say that the action of radiation is to give a certain number of the molecules excess energy. This excess energy may be energy of ionization, of excitation, or kinetic energy. By virtue of this excess energy the molecules are made more reactive, and hence chemical changes occur, the number of molecules participating being of the order of the number of ions produced.

Having concluded that the biological effect of radiation is due to chemical change, one has now to consider the extent of the region in which chemical change is induced by a single ionizing particle. This is synonymous with the extent of the region of ionization produced by the particle. In a gas at ordinary pressure the ions are initially distributed at distances of the order of 10^{-3} cm from the geometrical path of the particle, and may diffuse to considerably greater distances before disappearing by recombination. In media of the density of water the ions are initially distributed at distances of the order of 10^{-6} cm from the geometrical path of the particle and recombination is so rapid that very few survive sufficiently long to diffuse to greater distances. Thus the region of chemical change is to be identified with the initial distribution

of ions. This distribution has not been determined precisely by direct experiment, but may be inferred from experiments upon ionic recombination. Most of the data refer to gases, but provided the atomic numbers are not very different it is legitimate to apply the results to liquids, assuming that the scale of the distribution is inversely proportional to the density. For alpha particles the theory has been worked out by Jaffé (1913) while for beta particles a suitable extension of Jaffé's method has been made by one of the present authors (Lea, 1934). The ionization produced by an alpha particle is fairly homogeneous along the length of the track, and the function describing the ion density has thus cylindrical symmetry and is taken to be proportional to e^{-r^2/b^2} where r is the distance from the geometrical path of the particle and b is a parameter defining the scale of the distribution. The value of b required to fit ionization measurements in air is about 2×10^{-3} cm, from which a value of about 2×10^{-6} cm is to be anticipated for water or a medium of similar density. The ionization produced by beta particles (and therefore also by X-rays and gamma rays) is not homogeneous along the path of the particle but is localized in clusters of a few ions. A similar distribution function is assumed, viz., e^{-r^2/b^2} where r is now the distance from the centre of the spherical cluster and the value of b required to give agreement with ionization measurements is of a similar order to the value of b found for alpha particles. While the value of b deduced in this manner is probably a fairly accurate estimate of the mean distance of the ions from the centre of the cluster or column, the distribution function e^{-r^2/b^2} is arbitrarily assumed, and it would be wrong to deduce from it, for example, that an entirely negligible fraction of the ions are at distances greater than $3b$. The ionic density actually falls off with r at a slower rate, and there are occasionally ions produced at a distance of perhaps $100b$ (by fast delta rays). Thus while the greater part of the chemical change occurs within a distance of the order of b, an appreciable fraction may occur at distances several times greater.

3—The Poison Hypothesis

In the last section it has been argued that the lethal action of radiation is due to chemical change produced in the region in close proximity to the path of the radiation. In view of the fact that a large variety of chemical substances have a bactericidal action, the hypothesis immediately suggests itself that death is due not primarily to the radiation or destruction of essential constituents of the cellular protoplasm, but to the toxic action of the products of the chemical change taking place in the organism

itself. The lethal dose of radiation (10^4 to 10^5 *r* units) is sufficient, on the presumption that the number of molecules produced is about equal to the number of ions formed, to produce a concentration of poison of the order of one molecule for every 10^6 atoms. This concentration of a powerful chemical disinfectant is capable of producing death. Thus, considerations of concentration are not incompatible with the poison hypothesis. Further examination, however, makes it unlikely. The mechanism of chemical disinfection is not sufficiently understood for prediction to be made with any degree of certainty of the subsequent effect upon a bacterium of the intra-cellular production of an amount of poison not sufficient to cause immediate death, but the following arguments appear plausible. The concentration of the poison produced being determined by the ionization dosage, the fraction of the bacteria killed may be expected to increase with the time for which the poison is allowed to act. Thus the lethal action of a given radiation dose should be greater if administered slowly than if administered rapidly. Similarly, if the bacterial preparation is transferred to a nutrient medium immediately after exposure, the fraction dying might be smaller than if some time elapses between the end of the irradiation and the transference. Both these tests have been applied (p. 75), the results being negative. If the poison hypothesis is to be retained, it is probably necessary to modify it and to suppose that the poison produced is unstable and breaks down into harmless products either spontaneously or by the action of the bacterium, the concentration of poison falling rapidly to a negligible amount when the irradiation is discontinued. (This idea has been put forward by Pugsley, Oddie, and Eddy, 1935.) On this assumption it is easily seen that the concentration of poison present during irradiation is directly proportional to the rate at which ions are produced. The experiments already referred to indicate that the rate of death was accurately proportional (for a given type of radiation) to the rate of production of ionization, and thus leads to the conclusion that the rate of disinfection is accurately proportional to the concentration of poison. This result, while not impossible, is rendered unlikely by the fact that the majority of chemicals show a variation of rate of disinfection with concentration according to a power higher than the first.

Further evidence against the poison hypothesis is given by the experiments described subsequently (p. 73), in which it is shown that the rate of disinfection is independent of the temperature. The production of the poison by the radiation, being a molecular phenomenon, will be almost completely independent of the temperature, but the rate at which the poison acts may be expected to show the rapid increase with rise in

temperature exhibited by all chemical disinfectants. Thus temperature variation experiments are strongly opposed to the chemical poison theory.

A further objection to the poison hypothesis is the fact that the ionization dosage required to produce death is different for alpha and beta radiations. Chemical change would be expected to depend simply on the amount of ionization produced and not upon the nature of the particle producing it. Certain authors investigating the chemical effects of radiations have, however, reported differences in behaviour of alpha and beta particles even in simple inorganic reactions, which are equally difficult to explain, and pending the confirmation or otherwise of these results the dosage argument cannot be regarded as final.

Considering all the above arguments, it appears unlikely that the chemical poisoning theory is correct. It must be borne in mind however that in arriving at this conclusion from the experimental evidence it has been assumed that poison produced inside an organism by the action of radiation behaves in a similar manner to a poison introduced into the surrounding medium, so far as dependence of the rate of death upon concentration and temperature is concerned, and this assumption may possibly not be valid.

4—The Target Hypothesis and the Variability Hypothesis

Having provisionally rejected the theory that it is the appearance of the end product of the chemical change which causes death, one is left with the alternative that it is the disappearance of the initial product; in other words that molecules vital to the organism are decomposed. It may be imagined either that bacterial protoplasm is diffuse, destruction of a certain proportion of the molecules sufficing to cause death, or that there is in a bacterium an essential structure or nucleus in the biological sense which is specially sensitive, so that a given number of molecules decomposed in this target suffices to produce death, although the same proportion of decomposition elsewhere in the bacterium fails to do so. Purely bacteriological methods have up to the present been unable to decide definitely whether or not any structure exists of the type here envisaged, and it is necessary to look for decisive evidence between the two alternatives in the radiation experiments themselves. The assumption will first be made that no peculiarly sensitive region or target exists, and the implications of this point of view examined.

In the first place it is to be noted that the experiments on the rate of disinfection by known intensities of alpha and beta particles show that the duration of exposure required to produce death corresponds to the

passage of about 50 alpha particles through the bacterium or a considerable number of beta particles. Supposing death to ensue when an amount of ionization has been produced in the bacterium corresponding to the passage of n particles, the times required for this number to be reached will not be the same for different bacteria on account of statistical fluctuations. The fluctuations being of the order $n^{-\frac{1}{2}}$ of the mean time, will amount to about 15% for the alpha ray experiments and be quite small in the beta ray experiments. If n is a constant for all the individual bacteria exposed in an experiment, there will thus be a fairly definite exposure time required to produce death. Actually however the number of bacteria surviving diminishes gradually right from the start, showing that there are some bacteria which are killed by very small exposures, and some which survive exposures several times greater than the mean. This wide variation of survival times being much larger than can be explained as a result of statistical fluctuation it is necessary, if this hypothesis is to be retained, to assume that the bacteria vary in their resistance to radiation. The times of exposure required to produce death vary in corresponding manner, and may be taken as a measure of the resistance to radiation. The curve of distribution of resistance among the bacteria is obtained by differentiating the survival curve.

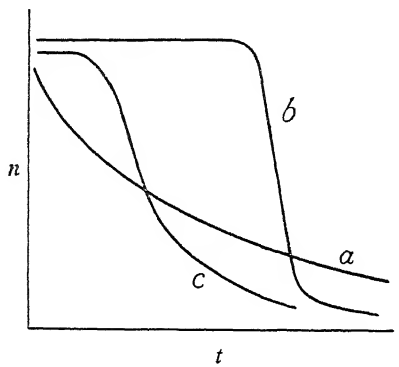

FIG. 1.

Fig. 1 shows a variety of hypothetical survival curves, n being the number of bacteria alive after irradiation for time t, and fig. 2 shows the distribution curves deduced from these, corresponding curves being similarly lettered. a, fig. 1, is the type of survival curve found experimentally; a, fig. 2 the corresponding distribution curve indicating that the most probable life of a bacterium under irradiation is zero. This is a very strange result and makes improbable the variability theory, though it must be remembered that a similar difficulty arises in chemical disinfection. The types of distribution curve which *a priori* would be most acceptable would be b or c, fig. 2, which are more or less symmetrical distributions about a most probable value different from zero. The authors have carefully investigated the initial portion of the survival curve both with alpha and beta rays, and have failed to find even a small flat portion such as is required by curves b and c.

Consider bacteria P, Q, R, whose resistances to radiation are repre-

sented by the points P_1, Q_1, R_1, on the distribution curve of fig. 3. Suppose irradiation occurs for a time t_1 sufficient to kill all the bacteria less resistant than P; after this irradiation the distribution will be represented by curve 2 of fig. 3. The surviving bacteria have clearly been damaged in some way by the radiation, since a bacterium which formerly had a resistance t now has a resistance $t - t_1$. It is plausible, biologically, that if

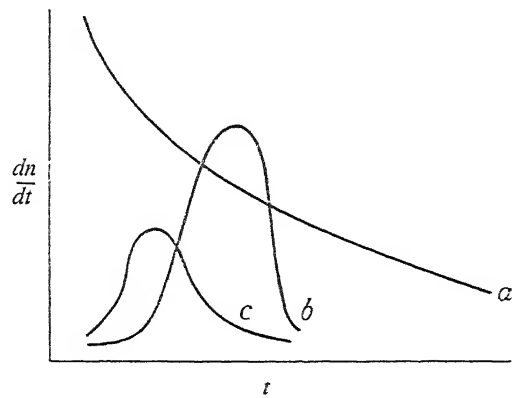

FIG. 2

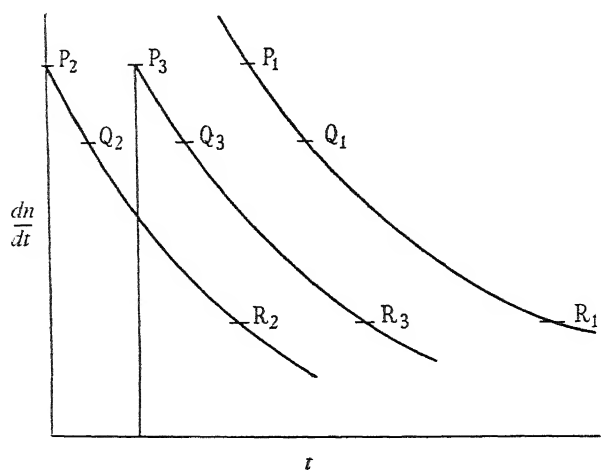

FIG. 3.

a bacterium which has been damaged is left for some time without further radiation, it will either die or will recover from the ill-effects. If, for example, partial recovery occurs, then the points P_2, Q_2, R_2 will move to positions P_3, Q_3, R_3. The bacteria which have been killed however cannot recover. The distribution curve after a partial recovery should thus be the curve 3 of fig. 3. The corresponding survival curve (fig. 4)

shows an initial horizontal portion. Experiments (p. 75) showed however no tendency either to recovery or death in an interval between two exposures. A further experiment along these lines which has not yet been tried would be to grow a culture of bacteria under constant irradiation, and by elimination of the weaker individuals perhaps obtain a distribution curve with a horizontal initial portion.

It appears, then, that the hypothesis that the bacterium is uniformly sensitive to radiation throughout its volume raises considerable difficulties, and attention will therefore be turned to the alternative hypothesis, that a target exists which is specially sensitive. Several authors have interpreted radiation experiments on bacteria and also on larger organisms in terms of the target hypothesis (Wyckoff, 1930; Herčik, 1934; Lacassagne

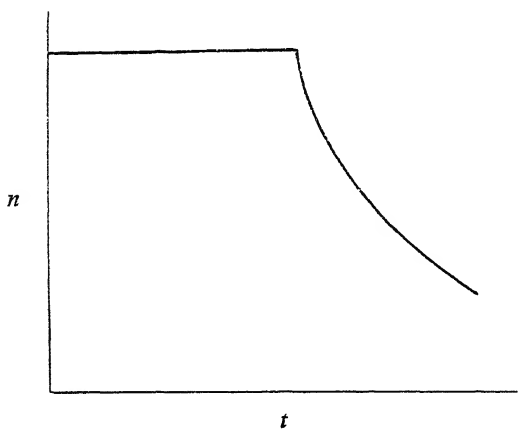

FIG. 4

and Holweck, 1934, and others). On this basis the shape of the experimental survival curve can be explained without the necessity of assuming a variation of resistance among different individual bacteria. The survival curve appears to be strictly exponential, $n = n_0 e^{-kt}$. The simplest mathematical property of the exponential function is that $\frac{dn}{n} \propto dt.$, *i.e.*, the probability of an individual bacterium dying in a given small time interval is independent of the previous duration of the irradiation. The simplest explanation of this simple result is that the bacterium is unaffected by the previous irradiation, *i.e.*, that death occurs suddenly as a single process and not as the climax of a gradual process. Then, supposing death to occur after a time of irradiation such that n alpha particles have traversed the bacterium, it appears that the first $n - 1$ were without effect and the nth instantaneously produced death. One is

led to the theory that there is a target in the organism, and one alpha particle traversing this target suffices to produce death of the organism. On this hypothesis, if n particles per sq cm per second traverse the bacterium and the survival curve takes the form e^{-nat} then a is the target area.

It may be hypothecated that to produce death more than one ionizing particle is required to traverse the target. Various elaborate derivations from first principles of the shape of the survival curve in this case have been given; a simple application of the Poisson distribution function however suffices. If the time of irradiation t is such that the mean number of hits on the target is m $(= nat)$, then the probability of the actual number of hits being x is $e^{-m} \cdot \frac{m^x}{x!}$. Supposing that r hits or more produce death, the probability of death in time t is $\sum_{x=r}^{\infty} e^{-nat} \frac{(nat)^x}{x!}$ and the fraction surviving is thus $\sum_{x=0}^{r-1} e^{-nat} \frac{(nat)^x}{x!}$. This function, for $r = 1$, is a simple exponential, for values of r different from unity the survival curve shows an initial horizontal portion which is relatively more pronounced the greater the value of r. Certain organisms exhibit a shape of survival curve compatible with the assumption that r is of the order of 40 (Crowther, 1926) but in their experiments with bacteria the present authors have obtained only exponential curves.

5—Methods of Testing the Target Hypothesis

The target hypothesis gives a simple explanation of the experimental type of survival curve, and the results of disinfection experiments may conveniently be expressed by stating the target areas. To prove that the target is a biological reality and not merely a convenient mathematical fiction it would be necessary to show that the target is a genuine property of the bacterium and not dependent upon other conditions of the experiment, such as type or intensity of radiation. The obvious experiment which suggests itself is to use several different intensities of alpha rays and beta rays and to calculate the target area in each experiment. Agreement between the calculated target areas in all experiments would lend strong support to the objective reality of the target. Suppose that the target area is equal for alpha and beta radiations and that death is produced by a single alpha particle or by a single beta particle. On this supposition the survival curves for both radiations would be expected to be exponential, the rates of death being equal when the numbers of alpha and beta particles per sq cm per second are equal. However, the ionization

produced in a given length of path is much more by an alpha particle than by a beta particle, and it may readily be imagined that while a single alpha particle traversing the target produces death, a number of beta particles will be required. Let this number be r; then the survival curve should take the form

$$\sum_{x=0}^{r-1} e^{-nat} \frac{(nat)^x}{x!},$$

where a is the target area and n the number of beta particles per sq cm per second traversing the bacterium. The survival curve for alpha ray disinfection would have the form e^{-nat}. If n is the same for the two radiations, the time required to reduce the number of survivors to 50% is about r times longer for beta rays than for the alpha rays; this affords one means of determining r. As remarked earlier the shape of the survival curve depends upon r, and an estimate of r can be made by this means without reference to the absolute rates of death. If the target is an objective reality these two determinations of r should agree.

The experiments of the authors on the point are described subsequently (p. 67). The value of r deduced from the time required to reduce the number of survivors to 50% was of the order of 1000; the value of r deduced from the shape of the beta ray curve was unity. Alternatively, one may express the result by saying that the exponential shape of the curves showed a single particle to suffice whether it was a beta particle or an alpha particle. The rate of disinfection then indicated the target area for beta particles was only a thousandth of that for alpha particles. This test therefore seemed conclusively opposed to the target hypothesis. In contrast to this result, all the other tests made pointed to the correctness of the target hypothesis, and made unlikely any of the alternatives, so far considered. The survival curves when two different intensities of alpha rays were used were both exponential: the times required to reduce the number of survivors to 50% being in inverse proportion to the source strengths (p. 74). This result is of course to be expected on the target hypothesis, the variation of source strength affecting n but not a in the formula e^{-nat}. Again, the rate of disinfection both by alpha and beta particles was found to be independent of the temperature (p. 73) a result to be anticipated on the target hypothesis and not on some of the alternative theories. A possible escape from the dilemma is discussed in the next section.

6—The Effect of Non-uniformity in the Distribution of Ionization

It has been pointed out by Mohler and Taylor (1934) that the target radius commonly obtained in bacteriological experiments is of the same order as the known radius of the columns of ionization produced by alpha and beta particles, and that the target area may thus represent the area of the cross-section of the column of ionization rather than the size of the sensitive region of the bacterium. The idea as it stands will not explain the beta ray target being much smaller than the alpha ray target area, since the radii of the columns of ionization are not very different. However, a more detailed examination in the light of the description of the distribution of ionization given in § 2 is more profitable. It was there pointed out that while alpha ray ionization is distributed fairly uniformly along the path of the particle, the ionization due to a beta particle is located in clusters at intervals along the path of the particle, the distance between clusters being many times the diameter of an individual cluster. If the target is larger than the distance apart of the clusters, the cluster structure will not produce any marked effect. If however the target is much smaller than the distance apart of consecutive clusters, there is a considerable probability that even when the path of the beta particle passes through the target, no ionization may be produced in the target owing to its falling in the gap between consecutive clusters. If b_1 is the radius of a cluster, and kb_1 the distance apart of clusters the probability of the target (considered much smaller than kb_1) falling on a cluster rather than between two clusters is $2/k$, which is quite small as k may be of the order of 100. The apparent target area to be inserted in the formula e^{-nat} for comparison with experiment is thus not πb^2 but $\frac{2}{k}\pi(b+b_1)^2$, where b is the true radius of the sensitive region, and b_1 the radius of the cluster. In the case of alpha rays no such cluster effect occurs, and the apparent target area is $\pi(b+b_2)^2$, where b_2 is the radius of the alpha particle column. The ratio of the apparent target areas for the two radiations is thus $\frac{2}{k}\left(\frac{b+b_1}{b+b_2}\right)^2$. Therefore the apparent target areas may be much smaller for beta rays than for alpha rays in qualitative agreement with experiment. The agreement cannot be expected to be quantitative, since as explained in § 2, the values of b_1 and b_2 and with them of k are rather indefinite.

It is seen that the true size of the sensitive region influences very

much the apparent target area for beta rays, which may be $\frac{2}{k}\pi(b+b_1)^2$ or $\pi(b+b_1)^2$ according as $b \ll kb_1$ or not, but much less so the apparent target area for alpha rays. Thus it is possible for two organisms to exhibit not very different apparent target areas as regards alpha ray disinfection, and very different target areas as regards beta ray disinfection. An indication of this effect was obtained; spores of *B. mesentericus* were compared with *B. coli* and showed a similar rate of death under alpha ray bombardment, and much slower rate of death under beta ray bombardment.

A lower limit can be fixed to the true size of the target by consideration of the ionization dose required to produce death, making the assumption that death is produced by a single ion pair. If N ions per cc per second are produced in the organism by the radiation, then in time dt the mean number of ions produced in the target is $Nvdt$ where v is the volume of the target (presumed spherical), and if death is produced by a single ion pair, $Nvdt$ is the probability of death occurring in time dt and e^{-Nvt} is the equation of the survival curve. Hence v can be estimated, and is found to be of the order of 10^{-17} cc (for spores of *B. mesentericus*) corresponding to a target radius of about $1 \cdot 5 \times 10^{-6}$ cm. An upper limit is provided by the apparent target area deduced from alpha particle irradiation experiments, and thus the target radius must have a value between $1 \cdot 5 \times 10^{-6}$ and $1 \cdot 5 \times 10^{-5}$ cm.

One possible explanation of the great difference between the apparent target areas deduced from alpha ray and beta ray experiment has already been described in the present paragraph: another explanation is possible which on the whole is preferable but which involves a new assumption. Death is supposed to occur when a certain minimum number of ions are produced in the target by the passage of a single particle. If fewer ions than the number ν are produced, then it is necessary to assume that no permanent damage ensues and the passage later of a second particle again producing fewer than ν ions fails to cause death although the total number of ions produced by both the particles may exceed ν. Practically every alpha particle traversing the target produces more than the minimum lethal number of ions, and then the apparent target area to alpha particles is $\pi(b+b_1)^2$ as before. Only a fraction of the beta particles traversing the target, however, produce sufficient ions to cause death.

The target is not sufficiently large to embrace more than one cluster of secondary ionization: the numbers of ions in clusters vary, the fraction of the clusters having a given number ν being a rapidly decreasing function of ν. Thus by choice of a suitable value of ν, the probability of the

passage of a beta particle through the target producing death can be made to have any value from unity downwards. The experiments of Ishino (1916) show that about one cluster in a thousand has as many as 30 ion pairs; thus the assumption that 30 ion pairs are required to be produced simultaneously in the target suffices to explain the difference between the apparent target areas to alpha rays and beta rays of *B. mesentericus* spores.

Similarly, the observed ratio of the apparent target areas of *B. coli* to alpha rays and beta rays (50: 1) can be explained on the assumption that in this organism 15 ion pairs are required to be produced simultaneously in the target to cause death.

7—The Validity of the Target Hypothesis

In view of the criticism which the target hypothesis has from time to time aroused it seems desirable to examine its foundations, and also to state the range of biological phenomena over which, in the authors' opinion, it may validly be applied. The principal argument which in the past has been advanced in support of the hypothesis is the simple explanation it affords of the observed shape of the survival curves of irradiated bacteria. On any other theory these curves have to be interpreted as indicating variation among the individuals of resistance to the radiation. The variability theory is adequate to explain a sigmoid shape of survival curve, but an exponential curve requires a very unlikely function for the variation of resistance of bacteria. However, if one is reluctant to admit that the exponential survival curves of bacteria under irradiation are to be explained on the variability theory, one is under the obligation to find some explanation other than variability of the exponential survival curves frequently obtained in chemical and heat disinfection experiments. Such explanations have been advanced, and while they have yet to be proved experimentally, one can say that there is no conclusive evidence that to any lethal agent bacteria exhibit the extreme skew form of variability curve which would be necessary to explain the exponential radiation disinfection curves on the basis of individual variation. On the other hand the form of the survival curve alone cannot be regarded as conclusive evidence in favour of the target hypothesis applying in any particular instance and other tests must be applied, such as proportionality of rate of death to intensity of radiation and the equality of effect of single and divided dose.

It is to be noted that the elaboration of the target theory developed in this paper makes it necessary to distinguish between the " true " target area and the " apparent " target area.

The latter is deducible directly from the rate of death and the intensity of radiation and may conveniently be used to express the result of any disinfection experiment with corpuscular radiation which yields an exponential curve, without prejudicing the issue as to whether the target hypothesis is valid or not. On the simple target theory the " apparent " target area is to be identified with the " true " target area (*i.e.*, the area of cross-section of the sensitive region of the bacterium): on the more detailed target theory developed above, however, the " apparent " target area may be either greater or smaller than the " true " target area. The " apparent " target area may be different for different radiations, but the " true " target area is independent of the radiation used.

The target theory has sometimes been criticized on the ground that it is absurd to attempt to explain in so simple a manner the action of radiation upon a living organism which is so complicated a chemical system. This criticism seems irrelevant. The changes taking place in the sensitive region after the production of ionization in it and which eventually lead to death, are doubtless highly complex. The target hypothesis is not concerned however with these changes: it depends simply upon the assumption that there is a sensitive region, the production of a small amount of ionization within which leads to death, while the production of a similar amount of ionization elsewhere in the organism does not have this effect. That regions of special sensitivity to radiation do exist in biological material has been demonstrated (Henshaw and Henshaw, 1933) and the additional postulate of the target theory, namely that there is only one such sensitive region, in an individual organism, is not improbable for bacteria. There is as yet not much evidence for supposing that the target hypothesis is applicable to organisms other than bacteria.

The target hypothesis is clearly only to be applied when the biological effect studied is some definite qualitative change such as death, or the loss of the power of reproduction, and cannot be extended to a graded action such as rate of respiration. It is not impossible, however, that the ionization, produced outside the sensitive region, may produce such graded action quite independently.

The target hypothesis is usually considered to explain not only the exponential type of survival curve, but also the sigmoid type. The experiments so far performed by the authors have not resulted in any sigmoid type curves being obtained. To explain the sigmoid curve it is necessary to suppose that when one particle produces insufficient ionization in the sensitive region to cause death, the subsequent and independent passage of other particles may, by their cumulative action, eventually be lethal. This assumption is directly opposed to the assumption which the

authors found it convenient to make in paragraph 6 to explain the difference in apparent target areas exhibited towards alpha particles and beta particles.

SUMMARY

Possible alternative mechanisms of the bactericidal action of alpha and beta radiations are elaborated, based upon the known physical phenomena accompanying the passage of these radiations through matter. The principal theories are:—

(1) secondary poisoning of the cell by chemical substances liberated in it by the radiation;
(2) destruction of essential constituents of the cellular protoplasm by the radiation;
(3) the " target hypothesis ".

Possible means of distinguishing between these alternatives are discussed. The results of the authors incline towards the target hypothesis. Possible explanations are discussed in detail of the marked difference in apparent target areas exhibited by certain organisms to alpha rays and beta rays.

II—Experimental, Alpha and Beta Particles

8—EXPOSURE TECHNIQUE

It has been usual in experiments on disinfection by radiation to employ the bacteria either in aqueous suspension or spread upon the surface of nutrient agar. In the present work it was thought advisable, in view of the small range of the polonium alpha particles (32 μ in water) to make use of thin films. An attempt was made to use liquid films obtained by dipping a platinum loop into an aqueous suspension of the organism, but it was not found possible to obtain stable, thin films of an area of 0·5–1·0 sq cm^2. In the method finally adopted thin gelatine films prepared in the following manner were used. A 10% solution of purified ash-free gelatine was inoculated and shaken vigorously for some minutes in a thermostat at 37° C. A platinum loop of diameter 0·9 cm made of wire of diameter 75 μ was dipped vertically into the suspension and slowly drawn out. The film so obtained had a thickness of from 20–50 μ depending on the speed of withdrawal of the loop from the gelatine solu-

tion. After drying for 10 minutes in a horizontal position most of the water had evaporated leaving a film of mean thickness of the order of 4 μ. The dried films usually showed interference colours near the centre, but were quite robust and remained stable indefinitely.

The initial weight of the gelatine film was found to depend upon the speed of withdrawal of the loop from the suspension, weight giving a smooth curve plotted as a function of the time of withdrawal. Rapid withdrawal yielded the heaviest films. By taking care that the measured time of withdrawal lay inside a suitable range, say 2·5 to 3 seconds, sufficient uniformity was obtained in the weights of the films. Frequent stirring of the gelatine solution was necessary. Settling of the organisms being presumably a slow process in the viscous gelatine suspension, the inference was that the film was mainly drawn from the superficial layer of the liquid.

The dried gelatine films were not of a uniform thickness, being thinnest in the centre, but the average thickness was much smaller than the alpha particle range. Irradiation of the film on one side only left untouched a small proportion of the organisms partly on account of shielding by the platinum wire and possibly also on account of the existence of a thickened portion of film near the edge. This was shown by the curve of number of survivors as a function of the time tending not to zero for very large exposures but to a finite number. Correction was therefore made for the shielded organisms by subtracting this number. The assumption that the finite limit of the exposure curve was indeed due to shielding was checked by an experiment in which the two sides in turn of the loop were subjected to prolonged exposure: the limit in this experiment was negligibly small.

After 10 minutes' drying a loop was placed in the exposure apparatus immediately above the shutter covering the radioactive source and the shutter opened for the required time. The loop was then dropped into 5 cc or 10 cc of water at 37° C and shaken for a few minutes, when the gelatine dissolved. Suitable dilutions were then plated in the usual manner, and counted in general after incubation at 37° C for 24 or 48 hours, but certain batches were incubated for 1 week in order to rule out any delayed growth. In the final experiments the concentration of bacteria was such that no further dilution was needed, thus minimizing errors of pipetting, and about 100 colonies per plate were obtained. Plates were always poured at least in duplicate.

This technique, while very convenient for some organisms was not so good for others. In the 10% gelatine suspension vegetative bacteria usually tended to multiply, and it was desirable to work in the lag phase;

while in the drying of the loops a large and somewhat irregular proportion of death occurred. Spores of *B. mesentericus* proved most suitable for the present technique since they did not suffer any change either in the gelatine solution or during drying. Some experiments were made with *B. coli* and *Staphylococcus aureus*, but suffered from the defects mentioned. On this account the detailed work has been done with the spores, and the data on the other two organisms are incomplete and less accurate.

The suspension of spores was made by scraping off the organisms from the surface of 5–10 agar plates, incubated for 1 week at 37° C, well shaking, heating for 15 minutes at 80° C to destroy any vegetative cells, centrifuging and making up to 50 cc. Such a suspension contained about 25×10^6 spores per cc, microscopic examination showing that it contained few, if any, vegetative cells, and could be kept indefinitely at 0° C. Suspensions of vegetative organisms were made up freshly as required from young agar slants. The culture of *B. mesentericus* was supplied by Dr. Graham-Smith of the Pathological Laboratory, Cambridge, giving a soft, easily emulsified growth in contrast with the adherent growths of some strains. *B. coli communis* Escherich was a stock culture from the Lister Institute, as also was the strain of *Staphylococcus pyogenes aureus* Rosenbach used.

9—The Radioactive Sources

Polonium deposited on a silver button 1·1 cm diameter was used as the source of alpha particles. A source of radium emanation provided the beta particles. To make the geometrical conditions as nearly comparable as possible the radon container was made in the form of a hemisphere of glass, the flat surface, of diameter 1·3 cm, being placed in the same place as that occupied by the polonium source in the alpha ray experiment. The thickness of the glass bulb (250 μ) was sufficient to absorb most of the beta rays from radium B and some from radium C: the number of beta rays emerging from the source per atom of radon disintegrating was taken to be 0·8. The beta rays were emitted from the active deposit on the walls of the hemisphere. If this is uniformly distributed, one-third will be on the flat surface and two-thirds on the curved surface. The solid angle subtended by the flat surface of the bulb at a point on the curved surface is on the average about $0{\cdot}4 \times 2\pi$. It follows that the active deposit on the curved surface is, from the point of view of the number of particles emerging from the flat surface, only 40% as efficient as active deposit on the flat surface. The mean efficiency taking into account active deposit on both flat and curved surfaces is 60%. Finally taking account of absorption it follows that for each atom of radon disintegrating in the

bulb, 0·24 beta rays emerge from the flat surface. For the alpha ray disc the corresponding result is, obviously, that for each polonium atom disintegrating 0·5 alpha particles emerge from the disc.

Knowing the number of particles emitted per second by the source, the rate of passage through a bacterium or " target " at the experimental distance from the source of about 2 mm can be evaluated.

Let N be the number of particles emitted by the source, uniformly distributed over a solid angle of 2π, and let the target be at O, distant d from the centre C of the source disc of radius s. For simplicity O is supposed on the axis of the disc, and the target is presumed spherical. The target radius r is much smaller than any other of the dimensions involved. The solid angle subtended by the target at the point P of the source (*see* fig. 5) is $\pi r^2/(d^2 + x^2)$ and the number of alpha particles hitting the target per second is thus

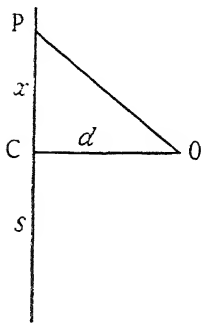

FIG. 5.

$$\int \frac{N2\pi x \,.\, dx}{\pi s^2} \cdot \frac{\pi r^2}{2\pi\,(d^2 + x^2)} = \frac{Nr^2}{2s^2} \log\left(1 + \frac{s^2}{d^2}\right).$$

This result will be an over-estimate if the target is not on the axis of the source, but provided the source is larger than the target (as in the present experiments) the error is small.

A second expression which can usefully be calculated is the rate of production of ionization (in air) by the radiations from the source at the point O.

If N is again the number of particles emitted by the source over solid angle 2π, and n is the number of ions produced per centimetre path in air, then the ionization produced per unit volume at O is

$$\int \frac{2\pi x\, dx}{\pi s^2} \frac{Nn}{2\pi\,(x^2 + d^2)} = \frac{Nn}{2\pi s^2} \,.\, \log\left(1 + \frac{s^2}{d^2}\right).$$

The strength of the polonium source was measured by placing it at one end of an evacuated tube 15 cm long, the other end of which was separated by a diaphragm from an air-filled ionization chamber, consisting of a pair of parallel plates 0·7 cm apart fixed parallel to the axis of the tube. The diaphragm was pierced by a pin hole covered by a thin gold foil of stopping power about 1 cm of air. The ionization produced by the narrow pencil of alpha rays defined by the pin hole was found by applying a field of 1000 volts per cm and measuring the saturation ionization current with a quadrant electrometer. Measurements were taken with known thicknesses of absorber covering the pin hole and by extra-

polation to zero absorber the total ionization deduced. Assuming the alpha particles emitted over solid angle 2π by 1 millicurie Po to produce a total ionization of $1 \cdot 33 \times 10^3$ e.s.u. the strength of the polonium source was deduced. The strengths of the radon sources were measured at intervals during their lives by comparing the ionization produced in an air-filled ionization chamber with the ionization produced by a radium standard, 2·5 cm of lead surrounding the source in all cases.

10—Disinfection Curves at Room Temperatures

Films containing spores of *B. mesentericus* were exposed to the radiation from a 3·67 millicurie polonium source for varying lengths of time, and the number of survivors counted. Several different loops were exposed for each different time of exposure, and the errors indicated in Table I are the standard deviations deduced from the mutual consistency of the counts made upon the different loops. A certain amount of error is of course inevitable on account of the statistical fluctuations in the numbers of colonies on the agar plates, and in the best experiments the standard deviation was no larger than was to be expected on this account alone. Generally, however, it was about twice as great, the additional error being due to a certain amount of variation in the weights of the individual loops, and other errors of experiment.

Table I—*B. mesentericus* Spores and Alpha Particles

Time seconds	Surviving fraction
0	1·00
3	0·86 ± 0·04
12	0·68 ± 0·04
20	0·52 ± 0·03
30	0·38 ± 0·03
40	0·26 ± 0·02
60	0·22 ± 0·02
80	0·16 ± 0·02
160	0·15 ± 0·02
240	0·11 ± 0·01
minutes	
90	0·09

The results are shown in Table I. The long exposure of 90 minutes indicates that about 10% of the organisms were out of reach of the radiation. When this amount is subtracted the number surviving is found to be an exponentially declining function of the duration of exposures

t, the half-period being 18 seconds. The curve of fig. 6 represents the function $0 \cdot 11 + 0 \cdot 89\, e^{-0 \cdot 693 \frac{t}{18}}$ and it is seen that this function fits the experimental results as closely as the indicated magnitudes of the experimental uncertainties permit.

As explained in Part I the exponential form of the function is evidence in favour of a single-hit target mechanism of disinfection; whether this be true or not it is convenient to express the result of the experiments in the form of a statement of "apparent" target area. The formula above leads directly to the result that the probability of a particular target being

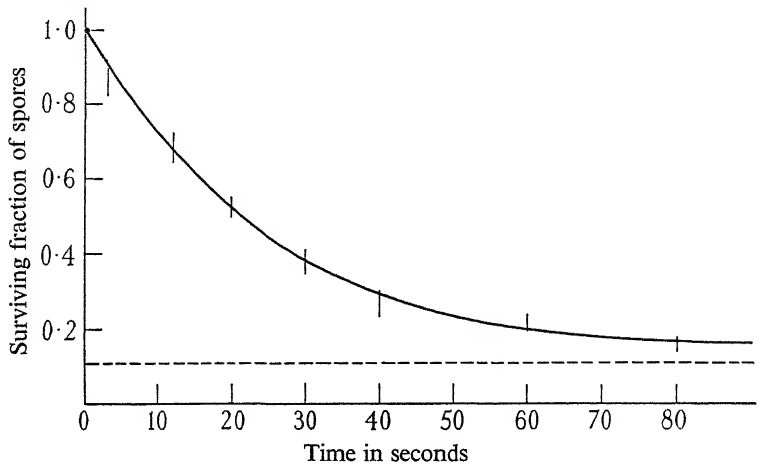

FIG. 6—*B. mesentericus* spores, α-rays.

hit in time dt is $0 \cdot 693\, dt/18$. In the nomenclature of paragraph 9 this same quantity is represented by $\dfrac{\mathrm{N} r^2 \log\left(1 + \dfrac{s^2}{d^2}\right) dt}{2s^2}$ where N is the rate of emission of alpha particles by the source over solid angle 2π, s is the source radium, and r the target radius. Using the values

$$\mathrm{N} = \frac{3 \cdot 7 \times 10^7 \times 3 \cdot 67}{2} \text{ per sec}$$

$$s = 0 \cdot 55 \text{ cm}$$

$$d = 0 \cdot 2 \text{ cm}$$

one obtains

$$r = 1 \cdot 3 \times 10^{-5} \text{ cm}$$

$$\pi r^2 = 5 \cdot 4 \times 10^{-10} \text{ cm}^2.$$

The measured size of the spores being about $2 \cdot 2 \times 1 \cdot 4\ \mu$ it is evident that the target area is appreciably smaller than the spore itself.

From paragraph 9 the rate of production of ions in air per cc per second at the point occupied by the target is

$$\frac{Nn}{2\pi s^2} \log \left(1 - \frac{s^2}{d^2}\right),$$

where n is the ionization density along an alpha track, ($= 3 \times 10^4$ ions per cm approximately).

Substituting the above values the rate of production of ions is found to be $2 \cdot 14 \times 10^{12}$ per cc per second, or $1 \cdot 02 \times 10^3$ r units per second. The survival curve being of the form $e^{-0 \cdot 693 \frac{t}{18}}$ *i.e.*, $e^{-\frac{t}{26}}$, the mean life of a spore is 26 seconds, and the mean lethal dose is thus $2 \cdot 6 \times 10^4$ r units.

Spores of *B. mesentericus* were irradiated by the beta rays from a radon source in a similar manner. The source had initially a strength of about 250 millicuries and decayed during the experiment. Allowance was made for the decay on the assumption that the effective exposure was proportional to the product of the source strength and the actual exposure. This assumption is justified by the result of the experiment described in § 13, in which sources of different strength were used. The exposure times in Table II are corrected to equivalent times corresponding to a uniform source strength of 200 millicuries.

TABLE II—*B. mesentericus* SPORES AND BETA PARTICLES

Corrected exposure time minutes	Surviving fraction	Number of loops
1·7	0·90 ± 0·05	14
2·5	0·84 ± 0·04	14
4·9	0·80 ± 0·04	7
9·8	0·69 ± 0·04	7
14·7	0·58 ± 0·06	5
27·7	0·47 ± 0·04	7
55·6	0·18 ± 0·02	5
85·7	0·06 ± 0·02	4

The fraction surviving is an exponential function of the corrected exposure time. In fig. 7 the experimental results are represented graphically, together with an exponential curve of half-value period 22·0 minutes, which is seen to fit the observation satisfactorily.

The target area and ionization dosage can now be calculated in a manner similar to the calculation already made for alpha rays. The source of 200 millicuries provides $7 \cdot 4 \times 10^9$ radon disintegrations per second, and by the calculation of § 9 each disintegration is accompanied

by the emission of 0·24 beta particles from the flat surface of the source. The quantity N to be substituted in the formulae therefore takes the value $1 \cdot 78 \times 10^9$ per second. The source radius s being 0·65 cm, one obtains for the probability of a beta particle traversing the target of radius r in time dt the value

$$\frac{Nr^2}{2s^2} \log\left(1 + \frac{s^2}{d^2}\right) dt = 5 \cdot 14 \times 10^9 \, r^2 \, dt.$$

The experimental survival curve being of the form $e^{-\frac{0 \cdot 693t}{22 \times 60}}$ or $e^{-5 \cdot 25 \times 10^{-4} t}$,

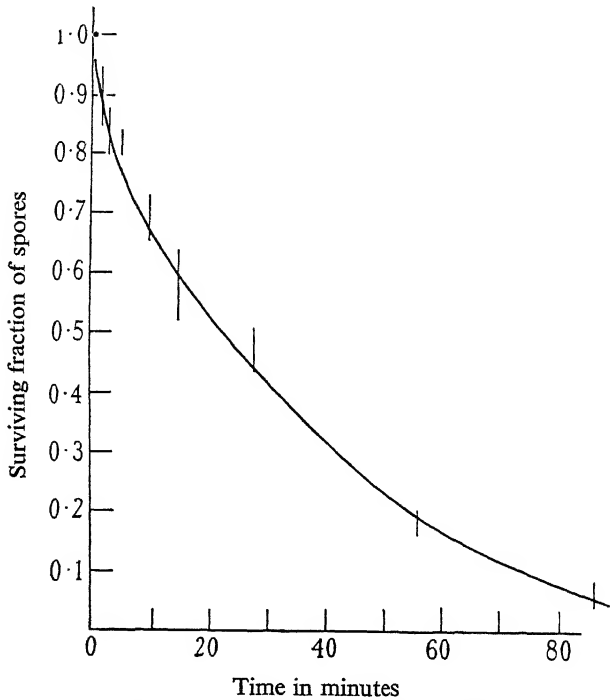

FIG. 7—*B. mesentericus* spores, β-rays.

a second expression for the probability of a beta particle traversing the target in time dt is $5 \cdot 25 \times 10^{-4} \, dt$. Hence one obtains for the target dimensions

$$r = 3 \cdot 19 \times 10^{-7}$$
$$\pi r^2 = 3 \cdot 20 \times 10^{-13} \text{ cm}^2.$$

The ionization dosage is calculated as before by the formula

$$\frac{Nn}{2\pi s^2} \log\left(1 + \frac{s^2}{d^2}\right)$$

n, the number of ions per centimetre track in air produced by the beta

rays, varies over fairly wide limits since the beta rays emitted by the source have all energies from zero up to about 2 million volts. The mean value assumed here is $n = 75$. One then obtains for the ionization produced in air at the place normally occupied by the target $1 \cdot 24 \times 10^{11}$ ions per cc per second, or $5 \cdot 92 \times 10$ r units per second. The half-value period of 22 minutes corresponding to a mean life of $1 \cdot 91 \times 10^3$ seconds, the mean lethal dose is $1 \cdot 13 \times 10^5$ r units.

Figs. 8 and 9 show logarithmic survival curves of *B. coli* and *S. aureus* under alpha particle bombardment. As explained in § 8, the dried film

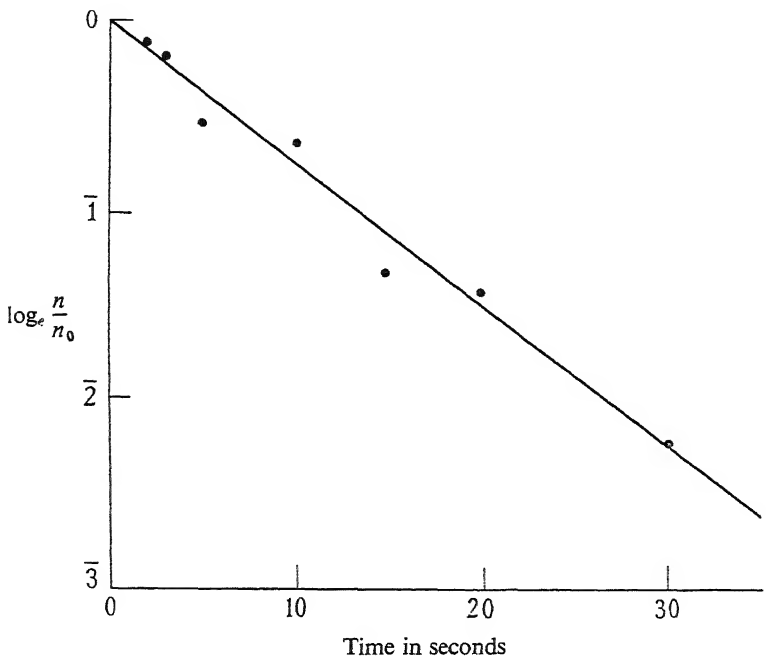

FIG. 8—*B. coli*, α-rays

technique is less satisfactory for these organisms than for the spores, and the results are less precise. The strength of the polonium source was in the experiments with *B. coli* 6·31 millicuries, and with *S. aureus* 5·59 millicuries. The half-value periods deduced from the exponential curves are 9·4 seconds for *B. coli* and 11·2 seconds for *S. aureus*. The target dimensions calculated in the same manner as before are:—

B. coli $r = 1 \cdot 4 \times 10^{-5}$ cm

$\pi r^2 = 6 \cdot 0 \times 10^{-10}$ cm^2

S. aureus $r = 1 \cdot 3 \times 10^{-5}$ cm

$\pi r^2 = 5 \cdot 7 \times 10^{-10}$ cm^2,

while the mean lethal ionization doses are:—

B. coli $2 \cdot 4 \times 10^4$ r units

S. aureus $2 \cdot 5 \times 10^4$ r units.

No beta ray experiments have so far been performed on *B. coli* and *S. aureus* in thin gelatine films, but an early experiment in which thicker

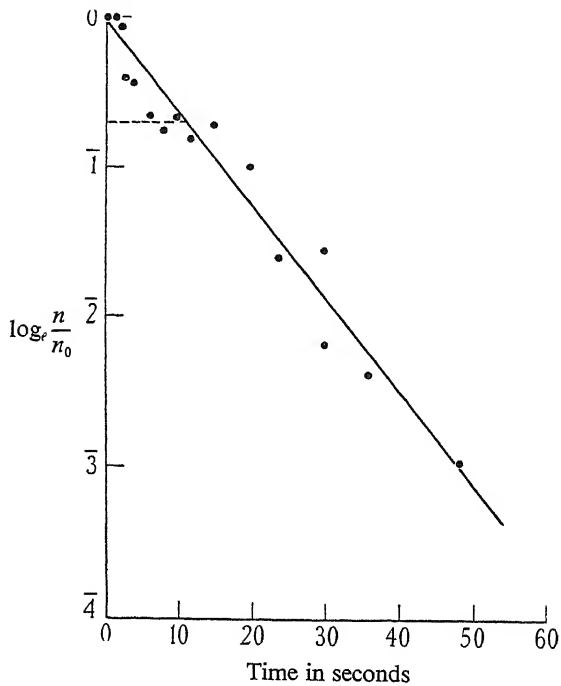

FIG. 9—*S. aureus*, α-rays.

liquid loops were used may be quoted. This yielded the results, approximately the same for the two organisms:—

target dimensions $r = 1 \cdot 6 \times 10^{-6}$ cm

$\pi r^2 = 10^{-11}$ cm^2

lethal ionization dose 4×10^3 r units.

It appears from this experiment that the target areas of *B. mesentericus* spores are very different from those of the other two organisms when

beta rays are concerned, but little different when alpha rays are employed. As explained, the experimental conditions were different from usual in the beta ray experiments on *B. coli* and *S. aureus*, but the result has been confirmed by subsequent experiments in which the three organisms were irradiated in aqueous suspension by radium gamma rays. This striking difference between the beta ray sensitivities of organisms equally sensitive to alpha rays may afford an interesting line of attack upon the mechanism of disinfection.

11—Temperature Variation

Experiments were made upon *B. mesentericus* spores by the usual film technique over a wide range of temperature. With alpha radiation a uniform exposure of 20 seconds was given at four different temperatures, with the results seen in Table III.

Table III—Temperature Variation: Alpha Rays

Temperature ° C	Fraction surviving
−50	0·51 ± 0·06
+20	0·52 ± 0·03
0	0·56 ± 0·03
−20	0·56 ± 0·03

It is evident that there is no appreciable variation with temperature of the rate of disinfection. A similar independence of temperature was observed when *B. mesentericus* spores were irradiated by beta particles, and also when *S. aureus* was irradiated by alpha particles. The data for *B. mesentericus* are shown in Table IV.

Table IV—Temperature Variation: Beta Rays

Corrected exposure minutes	Surviving fraction		
	+41° C	+20° C	−20° C
1·5	0·83 ± 0·08	0·95	—
7·5	0·81 ± 0·10	0·79	—
18·7	0·44 ± 0·05	0·55	—
17·5	—	0·57	0·57 ± 0·03
42·0	—	0·26	0·34
88·2	—	0·06	0·04

In contrast corresponding data are given for disinfection by a 0·2%

solution of $HgCl_2$ in contact with the spores for 5 minutes at different temperatures (Table V).

TABLE V—TEMPERATURE VARIATION: $HgCl_2$

Temperature ° C	Fraction surviving
+50	0·01
+20	0·33
0	0·54

12—MISCELLANEOUS EXPERIMENTS

Ideally, exposure experiments should be performed upon the bacteria with no irradiated matter in contact with the organisms. In the present experiments the bacteria were supported in a film of gelatine, and it is conceivable that chemical changes brought about in the gelatine by the radiation might be responsible for their death. An experiment was performed which negatived this suggestion. On films of sterile gelatine, prepared and dried in the manner ordinarily used for inoculated gelatine, drops of a suspension of *B. mesentericus* spores were placed and allowed to dry. Other films of sterile gelatine were exposed to the alpha rays for 5 minutes immediately before drops of the spore suspension were dried upon them. Finally all the films were dissolved and plated. The mean count on two films from the unirradiated gelatine was 73; and of four films of irradiated gelatine 70. It is thus clear that the presence of the gelatine has no disturbing effect upon the experiments.

An experiment was made upon the rate of death of *B. mesentericus* spores under two different intensities of alpha radiation, provided by polonium sources of 3·67 and 0·59 millicuries respectively. The half-value periods in the two experiments were 18 seconds and 100 seconds. Thus the ratio of the rates of disinfection was 0·18 : 1 and of the intensities of radiation, 0·16 : 1. It appears that over this range at least the rate of disinfection is proportional to the source strength, *i.e.*, the effect of a given ionization dosage is independent of the intensity of radiation. The experiment was tried of allowing an interval to elapse between completing the exposure and dissolving the film, to test whether any recovery or increased lethal effect occurred after irradiation had ceased. The result was negative. The effect of a divided exposure was also compared with the effect of a single exposure, again without any difference being found. The results are given below for alpha and beta irradiation of *B. mesentericus* spores. The exposure time in each case was chosen to reduce the number of survivors to about one-half of the initial number and

the results are expressed giving the ratio of the number surviving in each experiment to the number surviving in the experiment in which a single exposure was followed by immediate plating.

Alpha radiation.....	Single exposure of 20 seconds; immediate plating, 1·00. Single exposure of 20 seconds; plating after 10 minutes, 0·99 ± 0·08. Two exposures of 10 seconds; separated by 10 minutes, 1·02 ± 0·07.
Beta radiation	Single exposure 30 minutes; immediate plating, 1·00. Single exposure 30 minutes; plating after 50 minutes, 1·15. Two exposures of 15 minutes; separated by 50 minutes, 1·06.

The authors are indebted to Professor S. Russ and the Medical Research Council for radon, Professor J. Chadwick for polonium, and to Dr. Graham-Smith for cultures.

Summary

A technique is described for the preparation of bacteria in thin gelatine films for exposure to corpuscular radiations of small penetrating power. Experiments are described in which *B. mesentericus* spores; *B. coli* and *S. aureus* are irradiated by alpha and beta particles.

The fraction surviving is an exponentially diminishing function of the time of irradiation. The results are expressed in terms of mean ionization dosage required to produce death, and also by statement of the "target areas". The rate of disinfection is found to be independent of the temperature and for a given type of radiation to be proportional to the intensity of the radiation. No difference was observed between the effects of a divided exposure and a single exposure.

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The Influence of Fungal Decay on the Properties of Timber

I—The Effect of Progressive Decay by *Polyporus hispidus*, Fr., on the Strength of English Ash (*Fraxinus excelsior*, L.)

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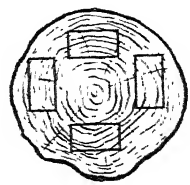
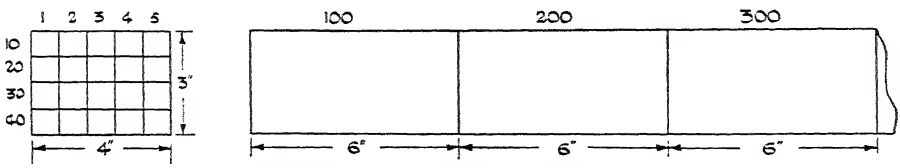
Introduction

The wood of English ash (*Fraxinus excelsior*, L.) is frequently found to break under a strain much below that which it can normally withstand, and a peculiar "brash" fracture is produced. This can, in some instances, be explained on anatomical grounds, for example, when the timber contains a large proportion of weak springwood. Cases have arisen, however, where ash wood, indistinguishable anatomically from material of good quality, is lacking in toughness and fractures suddenly under load in a "brash" manner. Nutman (1929) has found that in such timbers fungal hyphae are invariably present and suggests that *Polyporus hispidus*, Fr., which is one of the chief fungi attacking ash in this country, is mainly responsible for this "brashness". Little detailed work has, however, been carried out on the effect of this fungus on the strength of ash wood. Baxter (1925), who made a limited number of

tests on infected black ash (*Fraxinus nigra*, Marsh), found that incipient decay lowered the compressive strength of the wood. The lack of further and more comprehensive data prompted the present investigation on English ash specimens infected with *P. hispidus* under controlled conditions. In particular, it was considered that the effect of this fungus on the characteristic mechanical properties of ash wood, namely, toughness and elasticity, should be examined. Previous work at this laboratory (Cartwright and others, 1931) has shown that reliable results can be obtained in the testing of small specimens of wood after exposure to fungal attack, and that the progressive loss in mechanical strength which occurs can be closely correlated with the advance of the attack as evinced by chemical changes and loss in dry weight.



Conversion diagram of log



Allocation of bending specimens

FIG. 1—Conversion diagram of log and allocation of bending specimens.

MATERIAL

A log of English ash wood of good quality was sawn into baulks of approximately 4 inches by 3 inches. These baulks were marked off into lengths and divisions suitable for the preparation of small specimens* for static bending, compression, and impact bending (toughness) tests. This allocation, which supplied specimens for fungal inoculation and for controls, was similar to that used in the previous investigation (Cartwright and others, 1931). A conversion diagram is shown in fig. 1. The short

* The development of the small test-piece for mechanical testing of timber has been dealt with fully in a previous report by C. J. Chaplin, M.Sc., M.I.Mech.E. ("The Development of the Mechanical Testing of Timber in Great Britain"—'Congrès de Zurich,' 1931.)

lengths were taken in pairs from each baulk, namely, 100–200, 300–400, etc., so as to ensure correct end- and side-matching of the control test-pieces with those for inoculation. The specimens were carefully selected for straightness and evenness of grain and weighed before sterilization. The average moisture content of the test-pieces at conversion, obtained from control specimens, was 37·9%, based on oven-dry weight.

Experimental

Sterilization and Inoculation

The specimens, including controls, were sterilized by steaming at 100° C for one hour on each of three successive days. Those intended for the static bending and compression tests were exposed to fungal infection by placing them upon cultures of *P. hispidus* growing on 2% malt agar in a form of Kolle culture-flask provided with a reservoir in the neck to maintain moist conditions. The flasks containing the inoculated test-pieces were incubated at 22° C. The specimens for the impact bending (toughness) test were infected by placing them upon slope cultures of the fungus in glass tubes 18 inches long and 3 inches in diameter, which were kept in a culture-room at about 20° C.

Mechanical Tests

Two parallel series of mechanical tests were carried out on the experimental material, namely, (*a*) on specimens which had undergone decay, and (*b*) on the corresponding matched controls.

The controls were tested immediately after sterilization, and groups of decayed specimens at regular intervals, which varied for each of the three following types of test:

(1) *Static bending tests*, in which small test-pieces 5 inches in length and $\frac{3}{8}$ inch square in cross-section were used to determine the strength and stiffness of the timber under transverse loading applied without shock. A span of 4·67 inches was employed, the load being applied through a head, the rate of descent of which was 0·015 inch per minute. The general arrangement of the test is shown in fig. 2.

(2) *Compression tests*, from which the maximum crushing strength of the timber when loaded parallel to the grain was determined. Small bobbin-shaped test-pieces $2\frac{3}{4}$ inches long with the central part reduced to a $\frac{3}{8}$-inch square cross-section were employed. They were tested in a special compression cage, designed to give

parallel loading on the ends of the specimen. Fig. 3 shows the general arrangement of the test.

(3) *Impact bending or toughness tests*, in which the specimens were subjected to a suddenly applied transverse load, the property recorded being a measure of the toughness or resistance to shock. The test-pieces used were $\frac{5}{8}$ inch square in cross-section and

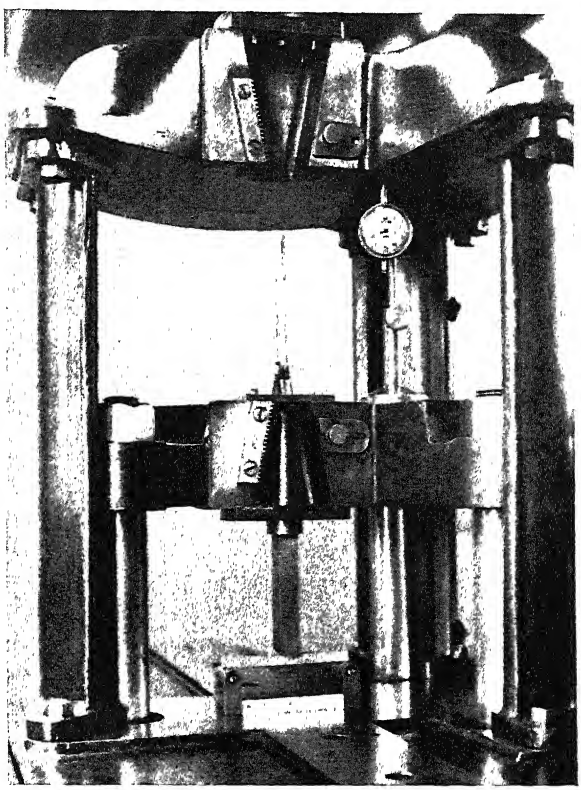


FIG. 2—Arrangement of static bending test.

10 inches in length. The toughness machine arranged for test is shown in fig. 4.

Moisture content, specific gravity, and ring-width determinations were made on each specimen in all three types of mechanical test. The results of the mechanical tests are recorded in Tables I–III. These include mean values for the decayed specimens in each type of mechanical test, together with the values for the corresponding controls. In addition to these actual test values, figures are given showing the reductions, at each period

of incubation, in ultimate bending strength, modulus of elasticity, maximum crushing strength and toughness, expressed as percentages of the control values.

Types of Fracture

An interesting feature of this investigation was the transition from the normal to the very " brash " or brittle fracture obtained in the static

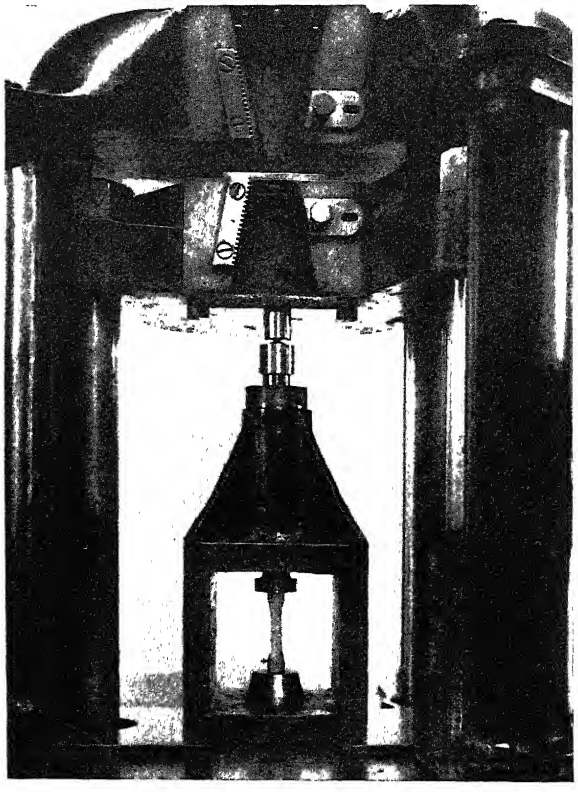


FIG. 3—Arrangement of compression parallel-to-grain test.

bending and toughness tests. In the early stages of infection a considerable shortening of the fibres was apparent after test on the side of the test-pieces subjected to tension in static bending. Signs of " brashness " were observed in specimens tested after 4 weeks' exposure to the fungus, and subsequently the extent of "brashness" increased until the fracture was entirely of a brittle nature. Similar results were obtained in the toughness tests, some " brashness " being observed after 2 weeks. In the compression tests the fungus attack did not greatly affect the type of fracture.

A further point of interest was the apparently sound condition of the specimens up to 16 weeks' incubation. Although the fractures were quite " brash ", up to this period the wood was still firm and hard to the touch, and, apart from some discoloration, there was no visible indication of weakness until the specimens were actually tested. It will be recalled that in a typical brown rot (Cartwright and others, 1931), external signs

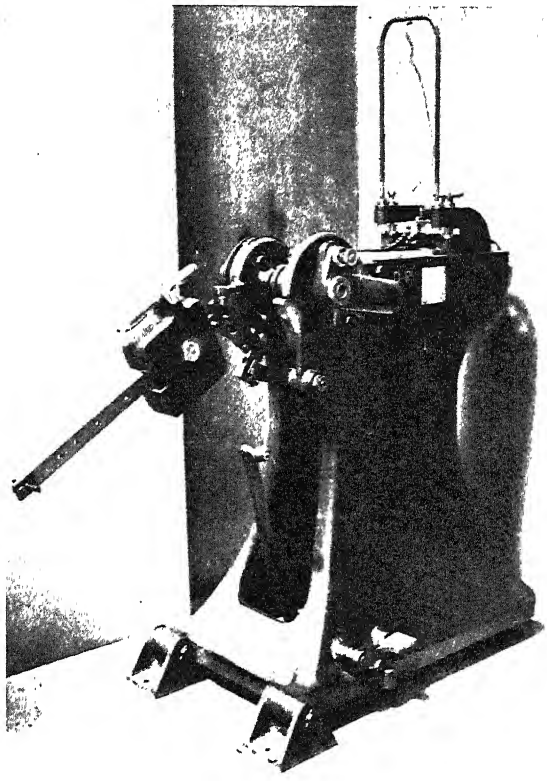


FIG. 4—Arrangement of impact bending or toughness test.

of decay were apparent even at a comparatively early stage of incubation.

Types of fractures are shown in figs. 5, 6, and 7.

Microscopical Examination

In each group of specimens two test-pieces were examined microscopically after mechanical testing. Microtome sections prepared from the parts bordering on the fracture were stained with safranin and picro-anilin blue (Cartwright, 1929), and the distribution and amount of fungus mycelium were estimated visually under the microscope.

TABLE I—*Static Bending*—AVERAGE VALUES FOR INOCULATED AND CONTROL SPECIMENS

Period of incubation weeks	Ring width inches		Moisture content %		Specific gravity oven-dry*		Maximum bending strength lb/sq in		Modulus of elasticity 100 lb/sq in		Reduction in bending strength †	Reduction in modulus of elasticity†
	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control		
Sterilized	0·10	0·13	41·9	40·8	0·546	0·546	9660	9800	1208	1178	1·4	−2·5
2	0·14	0·12	40·4	40·6	0·546	0·544	9110	9020	1127	1096	−1·0	−2·8
3	0·09	0·09	44·0	40·9	0·553	0·553	9260	9810	1149	1198	5·6	4·1
4	0·09	0·09	48·0	42·4	0·524	0·528	8690	8850	1034	960	1·8	−7·7
6	0·10	0·10	53·6	40·6	0·542	0·553	8140	8870	988	981	8·2	−0·7
8	0·13	0·12	51·7	43·3	0·528	0·548	7680	8670	1038	1036	11·4	−0·2
12	0·12	0·10	50·1	43·6	0·520	0·535	7560	8820	970	1034	14·3	6·2
16	0·10	0·10	62·1	40·5	0·511	0·549	7440	9450	1088	1180	21·3	7·8
20	0·12	0·11	51·8	41·9	0·515	0·552	7190	9090	1028	1047	20·9	1·8
24	0·11	0·11	47·2	46·2	0·496	0·536	6530	8820	946	1010	26·0	6·3
28	0·11	0·11	66·5	47·1	0·477	0·535	6260	8670	922	1034	27·8	10·8

* Based on oven-dry weight and volume as tested.

† Calculated as a percentage of the corresponding control value.

TABLE II—*Compression Parallel to Grain*—AVERAGE VALUES FOR INOCULATED AND CONTROL SPECIMENS

Period of incubation weeks	Ring width inches		Moisture content %		Specific gravity oven-dry*		Maximum crushing strength lb/sq in		Reduction in crushing strength†
	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control	
Sterilized	0·09	0·09	55·2	46·9	0·492	0·492	3720	3700	−0·54
2	0·10	0·11	45·8	48·8	0·527	0·528	4220	4130	−2·18
4	0·11	0·12	51·8	38·6	0·524	0·541	3890	4090	4·89
8	0·09	0·10	48·1	52·8	0·517	0·508	3590	3810	5·77
12	0·11	0·11	46·1	42·5	0·518	0·532	3500	3640	3·85
16	0·10	0·10	48·9	47·6	0·489	0·509	3200	3790	15·57
24	0·10	0·10	46·6	43·9	0·498	0·511	3240	3630	10·74

* Based on oven-dry weight and volume as tested.

† Calculated as a percentage of the corresponding control value.

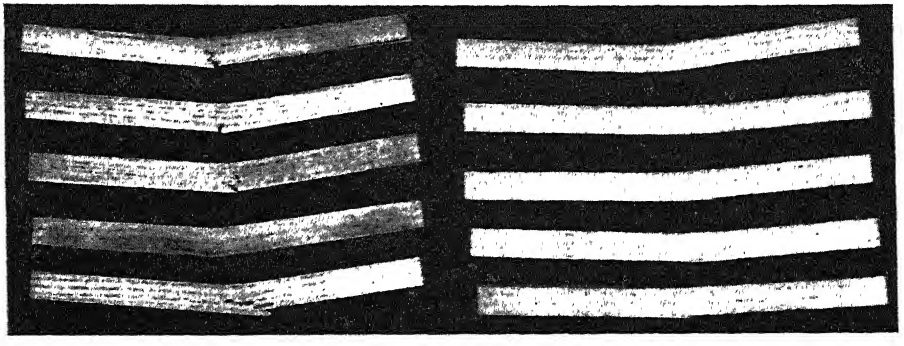
TABLE III—*Toughness*—AVERAGE VALUES FOR INOCULATED AND CONTROL SPECIMENS

Period of incubation weeks	Ring width inches		Moisture content %		Specific gravity oven-dry*		Toughness inch-lb		Reduction in toughness†
	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control	
Sterilized	0·06	0·06	46·4	51·2	0·509	0·491	273·5	252·2	−8·4
2	0·05	0·06	42·7	47·9	0·470	0·486	177·2	242·1	26·8
4	0·06	0·05	48·9	46·1	0·480	0·485	172·3	242·3	28·9
6	0·06	0·06	43·9	47·1	0·497	0·492	84·2	225·9	53·0
8	0·08	0·07	49·0	45·9	0·518	0·536	59·3	273·5	78·3
12	0·09	0·09	49·0	45·9	0·514	0·569	32·3	310·2	89·6
20	0·09	0·08	61·3	44·8	0·497	0·565	26·0	288·2	91·0

* Based on oven-dry weight and volume as tested.

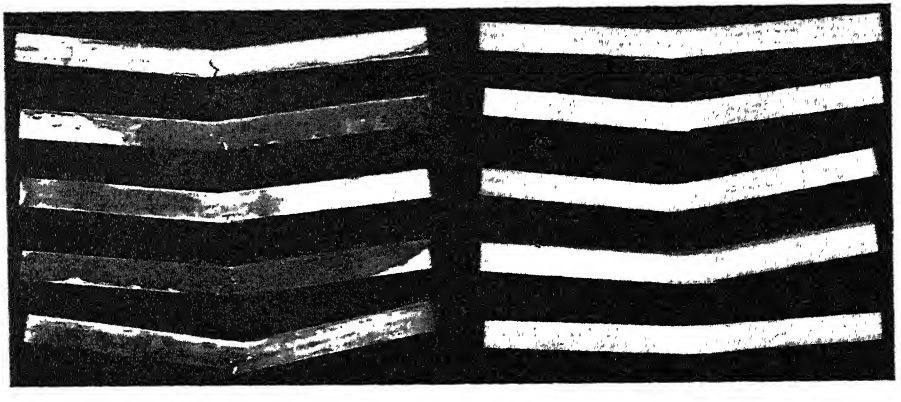
† Calculated as a percentage of the corresponding control value.

In the static bending specimens, the growth of the fungus was quite uniform and had reached the centre of the test-pieces examined after a period of only 2 weeks' incubation. After 4 weeks the fungal mycelium was evenly distributed throughout the specimens, being very plentiful in the vessels. Up to this time little penetration through the cell walls was



Ash specimens after 6 weeks' incubation. Sterilized ash specimens. Controls.

FIG. 5—Types of fracture of static bending specimen after 6 weeks' incubation.

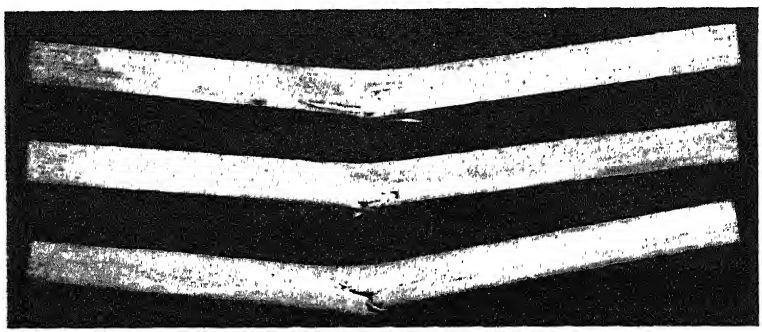


Ash specimens after 20 weeks' incubation. Sterilized ash specimens. Controls.

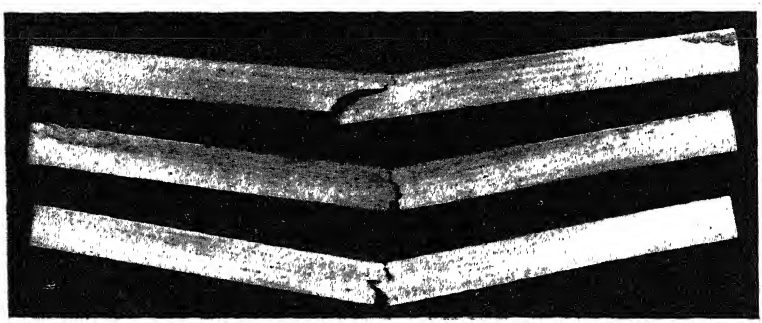
FIG. 6—Types of fracture of static bending specimens after 20 weeks' incubation.

observed, the hyphae for the most part utilizing the pits, but later, penetrations became frequent, and the amount of fungal mycelium appeared to remain more or less constant. The test-pieces appeared to be very little altered, no obvious signs of decay being seen. After 16 weeks' exposure, dark-coloured hyphae were observed, and the specimens showed signs of being seriously attacked, but it was not until about 28 weeks that any definite separation of the cells became apparent.

In the compression test-pieces the fungus did not make such uniform progress. After 4 weeks, hyphae were observed to be moderately plentiful throughout one of the specimens examined, but in another, little fungus mycelium was visible. After 8–12 weeks, hyphae occurred sparingly throughout the specimens, becoming moderately abundant throughout only after 16 weeks. It was not until about 24 weeks' exposure that the fungus was plentiful throughout. This may partly be



Ash specimens. Sterilized controls.



Ash specimens after 8 weeks' incubation.

FIG. 7—Types of fracture of toughness specimens after 8 week's incubation.

accounted for by the fact that owing to the shape of the samples, the central part of the test-piece was not in direct contact with the surface of the cultures as was the case in the static bending pieces.

In the toughness test-pieces, the mycelium was moderately plentiful throughout some specimens after 4 weeks, but little fungus was visible in others. Specimens examined at 6 and 8 weeks showed fungus mycelium to be plentiful throughout, and after 12 weeks, numerous bore holes were observed. At 20 weeks, fungal mycelium was very abundant throughout the specimens.

Chemical Examination

As in the previous study (Cartwright and others, 1931), the course of the decay in the inoculated material was followed by means of chemical analyses carried out at regular intervals. Experience has shown that the chemical composition of ash wood varies considerably according to the radial distance from the pith from which samples are taken,* so that in order to obtain reasonably representative analytical data for sound wood, duplicate analyses were carried out in the first instance on seven separate groups of sterilized controls selected at random. The average results for sound wood, which are shown in Table IV, therefore represent the arithmetic mean of 14 determinations for each component. The minimal and maximal values obtained for each component have also been recorded.†

From the decayed material, only those specimens which had been subjected to the static bending test were used for chemical analysis. Immediately after mechanical testing, each group of specimens was oven-dried to constant weight, and the loss in weight sustained by the group during decay was calculated. The material was then converted to sawdust, which was well mixed and screened to obtain sufficient 60–80-mesh flour for analysis. The analytical methods employed were essentially those of Schorger (1926), except that in the lignin determinations the digestion with 72% sulphuric acid was carried out at 10° C.

Discussion of Results

Consideration will first be given to the results of the static bending test, which are recorded in Table I. The relation between reduction in maximum bending strength and time of exposure to fungal attack is shown graphically in fig. 8, curve 1. As will be seen from this curve, the bending strength is immediately affected by the fungal infection, and it depreciates at a slightly diminishing rate until about the eighth week. The subsequent reduction continues at an approximately uniform rate of about 1% per week. At 28 weeks the depreciation in bending strength is of the order of 30%. It will also be observed from this figure that the rate of reduction in modulus of elasticity (curve 2) is very gradual and approximately uniform throughout the whole of the period of exposure to decay. The depreciation in this strength property is much less than

* Full experimental details concerning this conclusion will be published elsewhere.

† The authors are indebted to Mr. D. F. Packman for assistance in carrying out the chemical analyses.

TABLE IV—EFFECT OF PROGRESSIVE DECAY BY *P. hispidus* ON THE CHEMICAL COMPOSITION OF ASH WOOD

(All results expressed as percentages by weight of oven-dry, sound, sterilized wood)

	Sound wood			Decayed wood									
	Min	Average	Max										
ıration of decay in weeks..	—	—	—	2	3	4	6	8	12	16	20	24	28
›ss in weight	—	—	—	0·057	0·056	1·40	2·14	3·08	3·28	4·92	6·71	8·51	11·32
⁄ NaOH-soluble.....	22·29	23·91	24·62	22·86	24·37	23·25	22·31	20·72	20·73	20·85	20·13	21·19	20·21
:llulose	50·96	52·83	54·57	53·84	53·89	51·29	50·37	53·34	53·48	49·86	50·06	47·05	46·16
gnin	20·41	21·37	22·21	21·79	22·08	23·73	23·16	21·98	22·05	22·07	21·85	22·45	20·53
›tal pentosans	23·32	23·64	24·21	23·43	23·36	23·34	23·36	22·34	22·21	22·97	22·33	21·43	21·00
:ntosans in cellulose	10·75	12·04	12·99	13·02	13·10	12·00	10·58	11·77	12·13	10·83	11·61	10·06	9·33
:ntosans *not* in cellulose ..	10·33	11·60	12·75	10·41	10·26	11·34	12·78	10·57	10·08	12·14	10·72	11·37	11·67
›tal methoxyl content	6·29	6·38	6·68	6·37	6·71	6·52	6·18	6·54	6·17	6·02	5·88	5·88	5·67
ethoxyl in lignin	4·35	4·46	4·66	4·49	4·47	4·45	4·69	4·49	4·47	4·54	4·31	4·39	4·08
ethoxyl in lignin as % of lignin	20·33	21·00	21·39	20·63	19·15	17·48	19·67	20·40	20·28	19·35	19·71	19·58	20·20

that in bending strength; at 8 weeks' incubation the modulus of elasticity is only reduced by about 2%, and at the end of 28 weeks by 10%.

The mean values obtained in the compression parallel-to-grain test are given in Table II, and the relation between the maximum crushing strength and time of exposure to fungal attack is illustrated by curve 3 in fig. 8. The curve indicates a small and gradual loss in crushing strength over the total period of exposure, and a rate of loss approximately uniform. The reduction in crushing strength is less than that in bending strength, the depreciation in the former at the end of 24 weeks being 15%

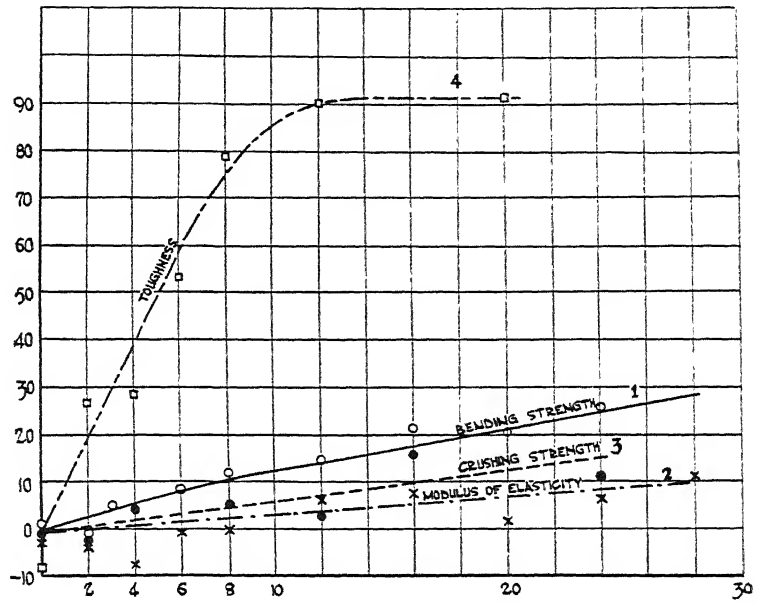


FIG. 8—Curves showing reductions in bending strength, modulus of elasticity, crushing strength, and toughness related to time of exposure to fungal attack.

and in the latter about 25%. Although this disparity may, in part, be due to the less vigorous growth of the fungus in the bobbins used in the compression test, it is largely explained by the fact that in the static bending test the maximum stress occurs in the outer fibres of the test-piece, where decay is more pronounced in the early stages of infection, whereas in the compression test the stress is evenly distributed over the whole section under test.

The effect of the decay on the toughness of ash wood is remarkably rapid; after only 2 weeks' incubation the reduction is as much as 20%. This is shown clearly by curve 4 in fig. 8. The subsequent rapid loss in toughness continues until about the eighth week, when the reduction is of the order of 75%. During the next 6 weeks the rate of decrease

diminishes. In the later stages of exposure the reduction in toughness is approximately constant, ultimately exceeding 90%.

In fig. 9 the percentage loss in dry weight is related to the time of exposure of fungal attack. The curve for maximum bending strength reproduced from fig. 8 is superimposed for comparison. It is of interest to note that nearly 5% reduction in bending strength occurs before any appreciable loss in dry weight takes place.

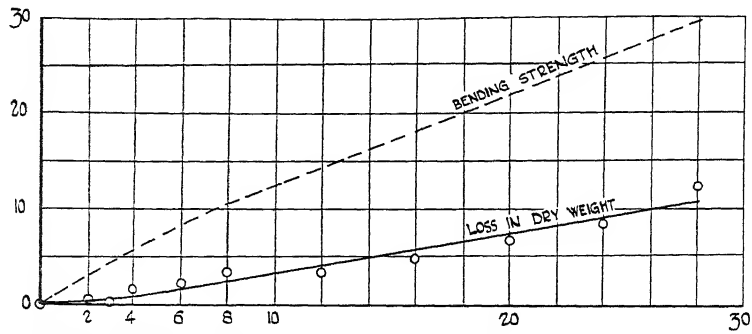


FIG. 9—Curve showing reductions in bending strength and dry weight related to time of exposure to fungal attack.

The ultimate chemical effect of *P. hispidus* on ash wood has already been indicated in previous work by one of the authors (Campbell, 1931). It has further been shown (Campbell, 1932) that, as a class, the white rot fungi are not consistent in the manner of their attack on wood. None of them has yet been found to have a specific action on the lignin in wood, and, moreover, there is no general uniformity with regard to either the order or proportion in which the major wood components are decomposed. The point of chief significance which has emerged from all the recent chemical work on the white rots is that cellulose and not lignin is the chief object of fungal attack.

This is substantially borne out by the analytical data presented here. It has already been indicated (Table IV) that loss in weight owing to decay is not significant until 4 weeks' incubation is reached, and it is only here that decomposition of cellulose and its associated pentosans becomes apparent. No other wood component appears to be affected, and the loss in weight is directly accounted for by the loss in cellulose, if the results for the decayed wood be compared with the average results for sound wood. The same is approximately true for 6 weeks' incubation. No such direct correlation between loss in weight and cellulose depletion is apparent, however, at either 8 or 12 weeks, although the data suggest that cellulose and pentosans are the main objects of attack. The com-

paratively high results for cellulose here might possibly be explicable on the assumption that in these cases the original test-pieces had comparatively high cellulose contents. At 16 weeks and onwards the depletion of cellulose is reasonably consistent. On account of the difficulty mentioned above of obtaining accurate matching of test-pieces on a chemical basis, it is impossible to illustrate the progressive depletion of cellulose by means of a smooth curve.

It seems that the pentosans, or rather the furfuraldehyde-yielding complexes, which are not associated with the cellulose, are particularly resistant to decomposition by the fungus. This has already been indicated in previous work (Campbell, 1931). The present series of analyses do not at first sight indicate with certainty, however, whether lignin is decomposed to any extent. Apart from the difficulties associated with sampling, the accurate determination of lignin in decayed wood is sometimes rendered difficult by the presence of fungus mycelium. For instance, it has been indicated by Thom and Phillips (1932) that, according to species, the mycelium of wood-rotting fungi may contain from 3·4% up to 54·08% of a lignin-like substance.

Wiertelak (1932) recognized this difficulty in his work on wood decayed by *Trametes pini*, but assumed that the mycelium of this fungus is completely insoluble in 72% sulphuric acid. He accordingly introduced a method of calculating the amount of fungus body present in the decayed wood, and applied the appropriate correction to the apparent lignin content of decayed wood. With certain reservations this method of correcting the lignin content may be of value. Wiertelak (1932) claims that by multiplying the methoxyl content of the lignin in the decayed wood by the ratio of lignin content to the methoxyl content of lignin in sound wood, he obtains the actual lignin content of the decayed wood. When the value for apparent lignin of decayed wood is greater than the calculated value, the difference is taken to represent the amount of fungus body present. This naturally implies the assumption that when lignin is decomposed by a white rot fungus, the main portion of the lignin complex and the methoxyl groups are depleted in such a way that the proportion of one to the other is not altered. Such an assumption, however, does not appear to be warranted by any experimental evidence. Further, in the light of the results of Thom and Phillips (1932) an accurate estimate of the amount of fungus body present in decayed wood cannot be obtained directly by Wiertelak's method. It would appear, however, that the application of Wiertelak's correction may be justified when the apparent lignin content of decayed wood is higher than the lignin content of sound wood, especially if there is independent evidence that the methoxyl

content of the apparent lignin, calculated as a percentage of the original wood, remains unaltered.

For instance, it will be observed from the analytical data, Table IV, that until the 20-weeks stage is reached, the methoxyl content of the lignin of the decayed wood, calculated as a percentage of sound wood, is substantially the same as that of the lignin of the sound wood. On the other hand, when the methoxyl contents throughout the series are calculated as percentages of the appropriate lignin contents themselves, the values for the decayed wood, after 3 weeks' incubation, are consistently lower than even the minimum value for sound wood. The lowest value is obtained at the 4-weeks stage. This happens to correspond with a relatively high apparent lignin content. It must therefore be concluded that after 3 weeks' decay, sufficient insoluble material is derived from fungal material to vitiate the lignin results. At the same time, it is evident that the amount of mycelium present approaches its maximum at about 4 weeks. For reasons already given, the actual amount of mycelium present cannot be calculated from the data, but it is interesting to note that if the lignin contents of the decayed wood be corrected according to the method of Wiertelak (1932) using the factor 4·79, the values are virtually constant until the 20-weeks stage is reached. Thereafter and until 28 weeks, the methoxyl groups in the lignin show signs of depletion, and correction cannot be applied to the apparent lignin contents for, obviously, if the methoxyl groups are the first components of the lignin complex to be depleted, the multiplication of the value for residual methoxyl by a standard factor would lead to low lignin values and at the same time, would indicate the presence of large amounts of mycelium in the wood. The obvious conclusion to be drawn from the data with regard to the effect of *P. hispidus* on the lignin of ash wood is that this component is not attacked at the immediate outset of decay, but that attack begins when the loss in weight of the wood is between 5 and 6%. The fungus therefore rightly belongs to group III of the white rots (Campbell, 1932).

It is observed that after 16 and up to 28 weeks' incubation, the loss in carbohydrates is insufficient to account for the total loss of wood substance caused by decay. As decay proceeds, this discrepancy becomes greater. It has been seen above that there is direct evidence of attack on lignin at 20 weeks, so that it must be assumed that if a reliable correction of the apparent lignin values, and possibly even of the cellulose values, could be made for the presence of fungus mycelium and/or by-products of the fungal reactions, after 16 weeks' decay, a substantial depletion of lignin would be revealed.

The experimental data show that as a result of decay, there is no substantial increase in the alkali-solubility of the residual wood substance. There may be a slight increase at 3 weeks, but thereafter the tendency is for the alkali solubility to decrease. This has been shown in the previous work (Campbell, 1932) to be a diagnostic chemical feature of the white rots of wood.

By an interesting coincidence, it happens that the loss in weight (11·32%) sustained by the wood after 28 weeks' decay is approximately the same (11·3%) as that previously obtained (Campbell, 1931) when sawdust was attacked by the same fungus for 12 months. The analytical results for both wood and sawdust are in good agreement, except that in wood attack on the lignin is less well marked. This may be due to the presence of more mycelium.

The chemical aspect of the effect of *P. hispidus* on the mechanical strength of ash wood may be summed up by saying that the loss in strength is caused largely by the depletion of cellulose and its associated pentosans, in the first instance, although as the attack proceeds depletion of lignin sets in, and the rate of loss in strength is maintained.

General Conclusions

There can be little doubt that the foregoing considerations derived from a combined mycological, mechanical, and chemical approach to the study of decay of wood caused by *P. hispidus* must serve to focus attention on certain fundamental, as well as practical, aspects of wood decay in general. The efficacy of the combined method of approach to a problem of such complexity as wood decay requires no emphasis, but that there is still room for improved methods for the ready diagnosis of decay in its incipient stages is a fact of paramount importance, which has emerged both from the present and the previous study (Cartwright and others, 1931). For instance, it has been seen, Table IV, that up to a period of 4 weeks' incubation, there are no external indications that decay has proceeded to any appreciable extent. The loss in dry weight is so small as to be almost insignificant, and yet the mechanical data indicate clearly that the bending strength of the wood begins to depreciate even during these early weeks of incubation. By this time the fungal mycelium, as revealed by microscopical examination, has penetrated to the centre of the test-pieces. From a purely mechanical point of view it must be concluded that this early reduction in bending strength is mainly due to a shortening of the wood fibres. If such were the case, it might be expected that supporting evidence would be obtainable from the chemical data

but, for obvious reasons, this is not forthcoming. From the theoretical standpoint the results of the mechanical tests could reasonably be explained by the partial transformation of cellulose into either oxycellulose or hydrocellulose, but a satisfactory evaluation of small quantities of such transformation products presents too great a difficulty at the present time.

Although the loss in bending strength at 2 weeks' incubation is only of the order of 2%, it is noteworthy that when the transverse-bending load is applied suddenly, the depreciation in impact bending strength or toughness at the same period is as much as 20%. It must therefore be concluded that in the early stages of the decay of ash wood by *P. hispidus*, toughness is the strength property which is most affected. The depreciation in the shock-resisting property of the timber before decay has advanced far enough to be recognized by ordinary inspection may not only be appreciable but indeed serious. The results of this investigation thus afford a large measure of support for the original contention of Nutman (1929) that incipient decay by *P. hispidus* is one of the main causes of "brashness" in ash timber.

As decay becomes more advanced, the results of the mechanical tests find ready explanation in the appropriate chemical and mycological observations, which latter are incidentally in close agreement concerning the amount and distribution of fungal mycelium in the test-pieces. The main source of fungal nourishment is the cellulose of the cell wall, and it is the breaking down of this component which is responsible for the deterioration in the strength properties of the wood. The ultimate depletion of lignin by the fungus serves to accentuate a condition of mechanical breakdown which is already well advanced.

Summary

The effect of the progressive decay of ash wood (*F. excelsior*, L.) by *Polyporus hispidus*, Fr. under controlled conditions has been investigated by means of parallel series of mechanical tests and chemical analyses.

It is shown that the bending strength of the timber is affected immediately by fungal infection, and that its elasticity or stiffness is only slightly impaired in the early stages of attack. The loss in crushing strength is small and gradual throughout the entire period of exposure to decay.

Attention is drawn to the serious reduction in toughness or shock-resisting ability caused by *P. hispidus*, even during the early stages of attack on ash wood, when decay is not sufficiently far advanced to be detectable.

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Some New Forms of Visual Purple Found in Sea Fishes with a Note on the Visual Cells of Origin

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[PLATES 4 and 5]

INTRODUCTION

This paper records the measurement of the absorption curves of a number of new forms of visual purple which have been found among fishes. For reasons which will be given immediately it was hoped that we could correlate these results either with the habits of the species or with the microstructure of their retinae, but the attempt was not successful. We believe the results in themselves to be important because they show that light-sensitive substances of varying absorptions can be found in the retina and it may not be impossible to discover substances which form intermediate links in the perception of colour.

The retinae of fishes have attracted considerable attention from histologists because of their large and easily studied visual cells and the extent of their photomechanical changes. The results reported here on the types, distribution, and behaviour of the visual cells in the species examined

are not claimed to be either exhaustive or final. The histology was carried out under difficulties and it was only possible to examine a few specimens of each species. It was found, however, that where other workers have also reported on the same retina (as in the elasmobranchs, eel, trout, etc.) the observations were always in agreement.

It was shown by Schultze (1866) that among animals which are active by night there is a predominance of retinal rods whilst in day animals the cones predominate. For this and other reasons it seems fairly certain that at levels of illumination below 0·1 metre-candles there is not enough light to stimulate the cones and that the rods with their contained visual purple are the essential intermediaries in the formation of a nervous impulse from the retinal image. Much of the evidence for this view is concerned with the properties of visual purple. This substance is bleached by light but its absorption of light and its rate of bleaching are different for different wave-lengths. If one compares a graph relating absorption or rate of bleaching of mammalian visual purple to wave-length, with a graph expressing the apparent brightness of different regions of a feeble spectrum, one is struck by the similarity of the two curves (Trendelenburg, 1904; Hecht and Williams, 1922). The conclusion to be drawn is that the apparent brightness of any feeble source of light varies directly with the energy absorbed by the visual purple. Now the light received by the eyes of fishes has to traverse a layer of water, sometimes of very considerable thickness. Since all waters absorb part of the light traversing them, and absorb some regions of the spectrum more than others, deep-water fishes must receive only a weak illumination, which is restricted to those regions of the spectrum which are least absorbed by the water. It would be reasonable to inquire whether there is any correlation between the make-up of the retina of a given species and the depth which it normally frequents.

The recent work of Arey (1932) has shown that in general fish living in deep waters or under conditions of feeble illumination have a predominance of rods in their retinae. In looking for a possible correlation between the type of visual purple and the histological structure of any given retina we had hoped, for instance, to find that a visual purple whose absorption curve was adapted to receive the wave-lengths available in deep waters, was derived from a retina in which there was a predominance of rods.

Köttgen and Abelsdorff (1896) described two forms of visual purple, one found amongst amphibia, birds, and mammals with the maximum absorption in the blue-green at a little more than 500 $m\mu$ and the other form found in fishes with its maximum absorption at 540 $m\mu$. Numerous

workers have confirmed the results at 500 $m\mu$ whilst Grundfest (1931) has found a maximum at 540 $m\mu$ for the sun-fish *Lepomis*. v. Studnitz (1932) claims to have discovered a retinal substance with a maximal absorption at 560 $m\mu$ in *Testudo graeca* but the result needs confirmation. Wald (1935) found that the maximum absorption of three marine fishes was at about 500 $m\mu$.

The Extraction of Visual Purple

The number of species which we investigated was limited because the fish had to be kept alive in complete darkness for some hours before use. The actual period varied from two to twelve hours, at the end of which time the fish were decapitated in a deep red illumination and the retinae removed. The most difficult part was the preparation of clear solutions suitable for spectroscopy. Many species gave solutions containing fat which was removed with difficulty. Most species had a vitreous of the same density as the solution, making the centrifuge ineffective, nor did it disappear on shaking as does mammalian vitreous. To overcome these difficulties the retinae were placed in saline and centrifuged lightly. The supernatant fluid was then pipetted off and replaced by distilled water to haemolyse the numerous red blood cells. The solution was again centrifuged and the retinae treated with 2% digitonin solution (Tansley, 1931) and allowed to stand for half an hour or so. The mass was then centrifuged and the supernatant solution of visual purple was filtered and if necessary centrifuged a second time.

The Photoelectric Spectrophotometer

The absorption curves in the visible spectrum were obtained by a specially built photoelectric spectrophotometer, fig. 1. In the design of this apparatus two points had to receive special consideration; the apparatus should be capable of dealing with very small quantities of solution and it was necessary for the apparatus to function with a light intensity which would cause no bleaching during the course of an experiment. Although designed for a special purpose we believe the apparatus gives results which compare favourably with those of any existing spectrophotometer.

The basis of the method consisted in passing a narrow spectral band through the unknown solution on to a photoelectric cell. The current passing through the latter was measured by a null-point electrostatic method which has been described by Moss (1930), and by Campbell and

Ritchie (1930). It will not be necessary to give a full description except of certain improvements which we have added.

A direct-reading spectroscope was used, the spectrum being focussed on an exit slit, and then passed through the solution under examination. As a source of light we used a 6-volt 24-watt head-lamp, the filament of which was focussed on the collimating lens of the spectroscope. This is a convenient method of ensuring that slight vibrations of the lamp will not change the quantity of light passing into the instrument. The lamp was run off accumulators, and the voltage supplied was controlled by a carbon

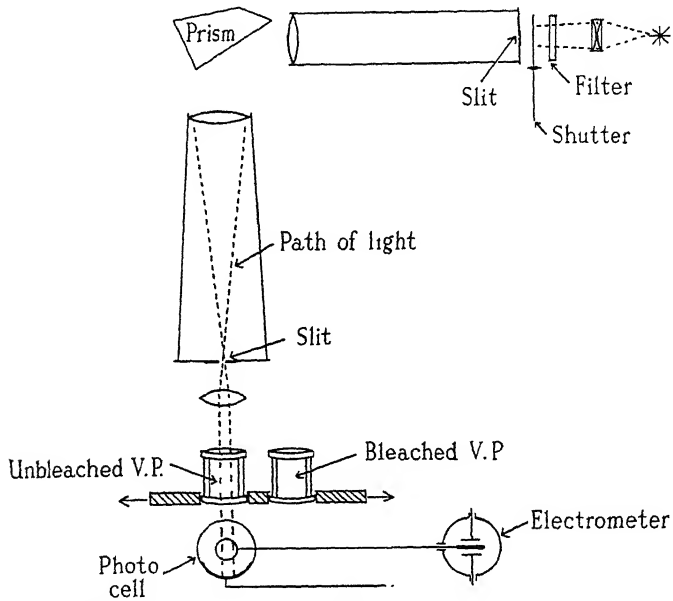

FIG. 1—Diagram of the general lay-out of the measuring apparatus.

resistance and measured by an open-scale voltmeter with leads running directly from the pins of the lampholder. We ran the lamp at 4·5–5 volts, that is, well below its rated voltage; even so the filament tended to sag slightly during an experiment. Variations in brightness can be partially overcome by inclining the filament at an angle of about 30° with the vertical, so that looking at the filament horizontally it appears of a more uniform brightness from top to bottom and the turns which are in front do not obscure those which are behind.

Scattered light within the spectroscope was reduced by colour filters which isolated a broad band of wave-lengths in the region under investigation. The filters chosen were Wratten 47; Ilford Micro 2; Wratten 58; a deep yellow solution of $K_2Cr_2O_7$ (3 parts) and $CuSO_4$ (2 parts);

Wratten A. The total breadths of the bands passed by the slits of the spectroscope were 1·5 $m\mu$ at 436 $m\mu$, 3·0 $m\mu$ at 546 $m\mu$, and 3·5 $m\mu$ at 579 $m\mu$.

The spectral band emerging from the exit slit, after being rendered parallel, was passed through the solution under examination to the photo-

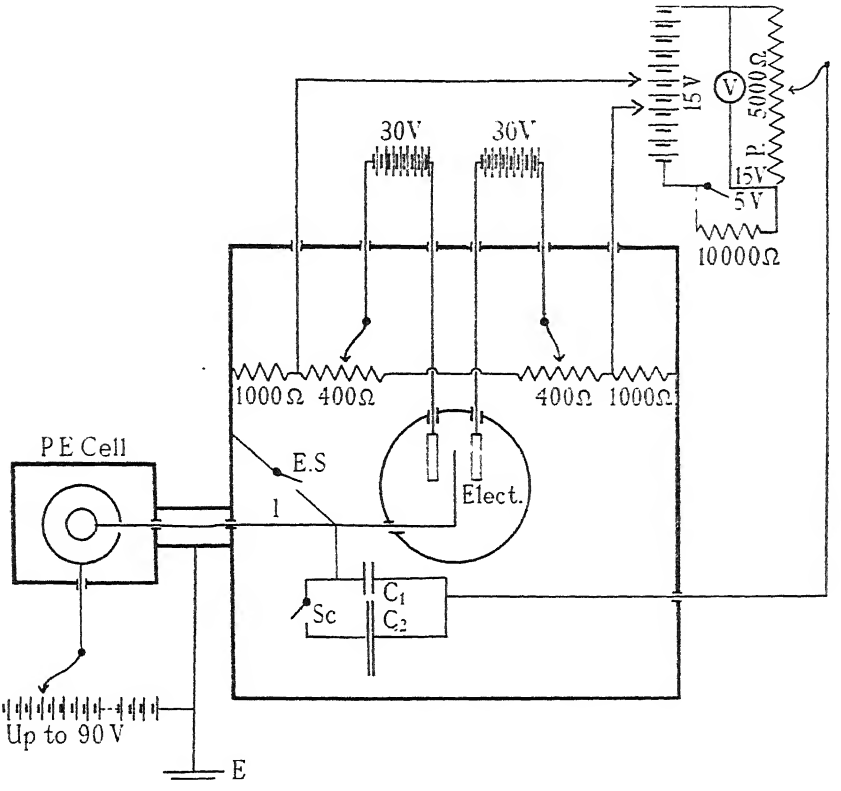

FIG. 2—Diagram of the electrical connexions. The screening boxes are shown by heavy lines. P.E. cell, photoelectric cell; I., incoming electrometer lead; P., potentiometer; Elect., Lindemann electrometer; E.S., earthing switch; Sc., condenser switch; C_1 and C_2, electrostatic condensers; E. earth.

cell. The capacity of each optical cell was about 0·5 cc and the length of path was 1·0 cm.

A sectored disc acted as a shutter and allowed the light to pass into the spectroscope for a constant time interval. This was rotated by a gramophone motor in conjunction with a spur reduction gear. It was adjusted so that the light passed into the spectroscope for 10 seconds and was cut

off for 20 seconds. The mean variation in the time interval was less than 0·1%.

The current passing through the photo-cell charges the insulated needle of a Lindemann electrometer, fig. 2. The potential on the needle is reduced to zero by inducing on it an equal and opposite charge through the condensers (C_1 and C_2). One plate of the condenser is connected both to the insulated needle and to the cathode of the photocell; the other plate is connected to a potentiometer so that when its potential is raised, the opposite plate becomes charged. With the earthing switch closed, the needle is at earth potential and occupies its zero position. Just before the light shines on the cell the earthing switch is opened, and later when the needle begins to move, it is kept at zero by means of the potentiometer. Insulation leakage is thereby reduced to a minimum. After the shutter (sectored disc) has closed, the needle is brought accurately to zero and the final reading of the potentiometer is made. It can readily be shown that the current and therefore the quantity of light are proportional to the potential delivered.

Very small quantities of light will apply only very small charges to the needle of the electrometer, and to obtain any reasonable accuracy it is necessary to decrease the deflexion of the needle for a given movement of the potentiometer. This can be done (*a*) by applying a smaller voltage to the potentiometer, and (*b*) by using a smaller charging condenser, so that a given change of potential applied from the potentiometer will induce a smaller charge on the needle of the electrometer. In the present apparatus arrangements were made for using both these methods, thereby permitting the accurate measurement of a large range of illuminations. It is not convenient to vary the time of exposure.

The complete circuit used is shown in fig. 2; in principle it is the same as the one described by Moss (1930). A screening box was mounted on the stage of a microscope. Inside this box were mounted the Lindemann electrometer, etc. The two condensers (C_1 and C_2) had capacities calculated to be about 7·4 $\mu\mu$F and 55 $\mu\mu$F respectively. By means of an insulated switch (Sc) it was possible to use either the smaller condenser or both together in parallel.

The potentiometer (P) was 46 cm long, wound on a straight former. It was mounted outside the box so that the observer at the microscope could operate it with his right hand whilst his left depressed the earthing switch. The voltage changing device on the potentiometer delivered either 15 volts or 5 volts to its ends. These voltages were kept constant with the aid of an auxiliary resistance and a voltmeter. In practice this voltage change was used in conjunction with the condenser changing device.

With the highest illuminations we used both condensers and 15 volts, and with the lowest, the small condenser and 5 volts.

The photocell was enclosed in an airtight case dried with P_2O_5. The screening box was dried with $CaCl_2$. Insulation of all vital leads was by sulphur or amber. It is also necessary to have a separate screen for the incoming electrometer lead (I) from the photocell within the screening box. Provided these precautions were taken the electrical side of the apparatus gave no trouble. It was found that good quality dry batteries were as good as accumulators and they were used throughout.

Drifts in the sensitivity of the apparatus, due to changes in battery voltages were eliminated by taking readings in pairs, each observation on the solution under investigation being immediately followed by an observation on a control solution.

The photocell was an Osram gas-filled type KMG7. The emission of this type of cell is not constant if it has previously been subjected to a high or low illumination. Such alternation in intensity would appear to be inevitable with our technique when the solution under investigation was very dense; the difficulty can be surmounted, however, by reducing the intensity of the incoming light used in the control measurements, so as to make the two illuminations on the cell more nearly equal. Caps with horizontal slits in them were made to fit over the condensing lens between the lamp and the spectroscope. They reduced the illumination on the slit without sharpening the image of the filament. The two caps reduced the illumination to 17·35% and 4·34% respectively.

The potentiometer (P) was calibrated against a standard instrument. The overall behaviour of the apparatus was checked by reducing the illumination of the photocell by known amounts and taking readings on the apparatus in the usual way. A series of sectored discs were made, the edges being provided by safety-razor blades. A photographic silhouette was then made of each disc and the angle of the open portion was measured by a vernier protractor. The discs were rotated in front of the entrance slit. The following figures were obtained. True transmission of sectored disc = 66·4%; reading obtained = 66·6%. True transmission = 25·0%; reading = 25·0%. True transmission = 9·77%; reading = 9·64%. True transmission = 3·425%; reading = 3·38%. These figures were the means of several readings taken on several days. It will be seen that the error is less than 0·5% when the difference in intensities of the lights falling on the cell is small. When the transmission being measured is of the order of 3%, the error is liable to be 1·5% of this figure. The inaccuracy in the measurement of small transmissions is the result of adaptation of the cell which was mentioned above. Accurate

readings can, however, be obtained by the use of one of the reducing caps.

When determining the absorption of visual purple one of the optical cells was filled with a bleached and the other with an unbleached solution of the substance. The two cells were then mounted on a changing mount in front of the photocell and readings were taken on them alternately. After a pair of readings the wave-length, etc., were changed. Apart from a blank period when these changes involved big changes in illumination, readings were taken from one end of the spectrum to the other, a reading at one wave-length occupying 1 minute. Although at first sight this appears to be a lengthy business, in point of fact it is rather quicker than visual methods of photometry, since with these, several settings must be made at each wave-length owing to the large mean variation of the readings. The present method is much more satisfactory when dealing with visual purple because the amount of light to which the solution is exposed is the same in all experiments.

Histological Methods

For each species investigated, sections were made of both the light and dark adapted eyes. For the light adapted eyes the fish was placed in a white basin of sea water in the direct sunlight at midday for an hour at least. The animal was then decapitated and the eyes removed in the light. Usually one eye was fixed in Zenker's solution and the other in F.W.A. (Flemming's solution without the acetic acid). In some of the fish with bigger eyes, such as the conger and mackerel, the isolated retina was fixed as well. In all cases at least one whole eye was treated in each fixative as we were anxious to study the behaviour of the pigment epithes lium as well as the structure and nature of the visual cells.

For the dark adapted eyes most of the fishes were kept in a tank in the dark room for 2–12 hours before decapitation. They were killed and the eyes dissected out in the dark by a deep red illumination. Fixation was also carried out in the dark.

The eyes were embedded in paraffin and the sections cut at 8 μ. The stains used were, Mallory's phosphotungstic acid haematoxylin and Mallory's triple connective tissue stain after Zenker's, and these two stains together with Heidenhain's iron haematoxylin after F.W.A. fixation. One eye of most of the species was fixed in 2·5% platinum chloride after dark adaptation to demonstrate the presence of visual purple. In the case of some of the bigger eyes with very tough outer coats, such as the mackerel, pollack, and gurnard, it was found necessary to remove the

sclera before cutting the sections. This was done during the paraffin impregnation. Before putting the eyes into the last change of paraffin they were allowed to cool, and it was found that in this way the sclera and tapetum could be removed without destroying the relation between the retina and pigment epithelium. The eyes were then returned to the oven and embedding completed in the normal way. Before removal from the head some of the eyes were subjected to the light of a 60-watt opal bulb at 12 cm distance for 15 minutes after decapitation in the dark. It was found that light had no effect on the movements of the pigment or visual cells after decapitation (*see* Arey, 1916).

Spectroscopic Results

These are given in Table II. Curves for three fishes are shown in fig. 3.

Of the 12 species examined by us no two had coincident maxima for the absorption curves of their visual purple solutions. The maximum could lie anywhere between 505 $m\mu$ and 545 $m\mu$. The determination was made on plaice four times, on pollack twice, on two closely related species of ray and on other species once. It was always found possible to repeat the result within 2·5 $m\mu$. Innumerable curves on frog and rat visual purple have all had identical maxima (about 505 $m\mu$). It seems certain therefore that the differences in the maximum absorption are outside experimental error and that there may be as many varieties of visual purple as there are genera of salt water fish. In view of the finding of only two types of visual purple by Köttgen and Abelsdorff (1896), this statement must be examined carefully. We have confirmed these workers' results on frogs, rats, trout, and tench and we have no doubt that their other results hold. Some significance may be attached to the fact that on their first run with *Perca fluviatilis* they found the maximum absorption at 520 $m\mu$, but they could not confirm the result. It is noteworthy that all fishes used by Köttgen and Abelsdorff frequent fresh water; with ocean living species things appear to be quite otherwise.

It is possible that the retina of a fish such as the plaice contains a mixture of two types of visual purple having individual maxima of absorption at 505 $m\mu$ and 540 $m\mu$ which when combined give a maximum at 520 $m\mu$. We did in fact obtain slight evidence for the simultaneous existence of two forms in the retina of the pollack. Distilled water alone does not cause any considerable liberation of visual purple from the rods of frogs and rats. In fish, however, it soon became obvious that the washings in distilled water designed to remove the blood, led to a liberation of a light-sensitive substance in addition to the usual liberation with digitonin.

In pollack the water-extracted substance had a maximum absorption of 525 $m\mu$ whereas the maximum absorption of the digitonin solution was at 535 $m\mu$. It must be confessed, however, that the water solution was spectroscopically undesirable, although it gave a good symmetrical curve. We did a similar run on trout visual purple but found that both the water and the digitonin extract had maxima at 540 $m\mu$.

In computing the reliability of the results another factor must be considered, namely the presence of yellow substances contaminating the solutions. It was found that there was a progressive difference in the density between the bleached and the unbleached visual purples during the course of a set of readings. This was shown to be due to the progressive loss in colour of the yellow substances in the bleached solution, a process which occurs in the dark (Hosoya, 1934). Similar changes have been investigated by Hecht and Chase (1934) and others. Since it is a blue-absorbing substance which disappears, the readings at the shorter wave-lengths show a greater increase than those at the longer wave-lengths. This has the effect of moving the apparent maximum of absorption of visual purple towards the blue end of the spectrum. The shift is quite small however since the edge of the absorption band of the yellow substances is very blunt. If it were possible to make a full correction for these substances, it is probable that the absorption curve of visual purple would be roughly symmetrical when plotted to a scale of wave-lengths.

Histological Results

The histological results are given in Table I.

Table I—Summary of Results

Elasmobranch

Dogfish (*Scyllium canicula*)—Maximum absorption at 505 $m\mu$. Salt water fish living at 1–100 metres. Active at night (Franz, 1905). Medium yield of visual purple. Unpigmented epithelium with rods only.

Ray (*Raia clavata*)—Maximum absorption at 510 $m\mu$; (*Raia maculata*) maximum absorption at 510 $m\mu$. Salt water fish living at 10–200 metres. Active at night (Franz, 1905). Low yield of visual purple. Unpigmented epithelium with rods only (fig. 4, Plate 4).

Teleost

Eel (*Anguilla vulgaris*)—Maximum absorption at 505 $m\mu$ (*see also* Kühne and Sewall, 1879, on rate of bleaching). Fresh water form. Active at night (Krause, 1886, and our own observations). High yield of visual purple. Rods and cones (single only). Rods and pigment move in response to dark and light adaptation but cones remain stationary.

TABLE I—(continued)

Teleost—(contd.)

Mackerel (*Scomber scomber*)—Maximum absorption at 505 $m\mu$. Salt water fish living at 0–120 metres. Sight feeder in day. Medium yield of visual purple. Rods and cones (twin). Visual cells show photomechanical movements but pigment did not advance in light adaptation (animal was, however, anoxaemic) (figs. 7 and 8, Plate 5).

Dragonet (*Callionymus lyra*)—Maximum absorption at 510 $m\mu$. Salt water fish living at 20–300 metres. High yield of visual purple. Rods and cones (some twin). Normal photomechanical movements of visual cells and pigment.

Plaice (*Pleuronectes platessa*)—Maximum absorption at 520 $m\mu$. Salt water fish living at 2–100 metres. Does not use eyes in day. Medium yield of visual purple. Rods and cones (some twin). Visual cell movements very small if present at all. Pigment movements normal (fig. 6, Plate 4). The dab (*Pleuronectes limanda*)—A salt water fish of similar habits but uses its eyes more. Visual cells, etc, similar to plaice. Absorption curve not done.

Wrasse (*Labrus bergylta*)—Maximum absorption at 525 $m\mu$. Salt water fish living at 10–20 metres. Sight feeder by day. Medium yield of visual purple. Rods and cones (mostly single). Normal photomechanical movements of visual cells and pigment.

Pollack (*Gadus pollachius*)—Maximum absorption at 530 $m\mu$. Salt water fish frequenting in-shore waters. Medium yield of visual purple. Rods and cones (mostly twin). Normal photomechanical movements of visual cells and pigment (figs. 9 and 10, Plate 5).

Conger (*Conger vulgaris*)—Maximum absorption at 535 $m\mu$. Salt water fish living at surface and very deep. Probably uses eyes at night. Low yield of visual purple (difficult extraction). Rods only, the pigment advances in light adaptation; it is not certain whether the rods move or not (fig. 5, Plate 4).

Tench (*Tinca vulgaris*)—Maximum absorption at 537 $m\mu$. Fresh water fish going deep during the day. High yield of visual purple. Rods and cones (probably some twin). Cones and pigment show normal photomechanical movements; doubtful whether rods move at all.

Trout (*Salmo fario*)—Maximum absorption at 540 $m\mu$. Fresh water form living in shallow water; sight feeder by day. Low yield of visual purple. Rods and cones (many twin). Normal photomechanical movements of pigment and visual cells.

Gurnard (*Trigla hirundo*)—Maximum absorption at 545 $m\mu$. Salt water fish living at 20–200 metres. High yield of visual purple. Rods and cones (some twin). Cones advanced outwards in dark adaptation. No data for light-adaptation movements.

The investigation on the elasmobranch retinae reveals nothing new. One point, however, may be mentioned here. It has been found that for the mammalian retina, fixation with F.W.A. and other osmic acid solutions gives quite a different structural picture from that given with ordinary

fixatives such as Zenker and Bouin and their modifications. The structure of the visual cells and more particularly of the inner layers of the retina are clearer with F.W.A. We found that while this difference was also noted in the elasmobranch retinae, no advantage was gained by using osmic acid fixatives on the teleost retinae. In view of the theory that the mammals are more nearly related to the elasmobranchs than to the teleosts this finding is of some interest.

In general the elasmobranch retinae were found to be composed mainly of rods though a few cones were recognized in one specimen of *Mustelus vulgaris* examined, as reported by Franz (1905), and Schaper (1899). The absence of cones in the retina of *Scyllium canicula* was also found by Franz, Garten (1907), Verrier (1930), and others, while Franz and Verrier agree that there are rods only in *Raia clavata*.

The position when we turn to the Teleost fish is not so simple. The two members of the family Anguillidae examined are of special interest. Not only do they produce visual purple with very different absorption bands but their retinae show quite different pictures. Although one member is usually a fresh water and the other a salt water inhabitant, it is probably fair to say that their general habits are very similar. Both are nocturnal and both have retinae rich in visual purple. Garten (1907) found that while the rods and pigments of *Anguilla vulgaris* move in response to changes in adaptation, the cones remain stationary. This retina appears to be unique in this respect. No double cones were found in this eye (*see* Friis, 1879, and Krause, 1886). Although the fresh water eel retina is well supplied with cones none is found in the conger retina. This does not necessarily mean that cones are entirely absent from the conger eye as a positive result is obviously more certain than a negative in this respect; but it does show that the cones, if present, must be so scarce as to be of no functional significance to the fish. This eye seems to be the only one among the teleosts so far examined to have a practically pure rod retina.

The results given by the mackerel in regard to the apparent lack of movement of the pigment epithelium are unusual and surprising. Unfortunately only one fish was used for the light adapted preparations. This had been in a shallow white basin in the boat from which it was caught for several hours before it was brought ashore and killed. The cones are well retracted but the pigment had not advanced to cover the rods at all. The fish was anoxaemic when brought in though the heart was vigorous. This may account for the sluggishness of the pigment, as Arey (1916) found that the pigment does not move in the absence of the attachments of the eye even if the optic nerve is intact. He suggested

that the circulation to the eye as opposed to the retina was necessary. On the other hand he also found that variations in the amounts of carbon dioxide and oxygen present had no effect on the normal movements so long as the animal lived. Friis (1879) claims to have found only twin cones in the mackerel retina; although it is not possible to deny the existence of single cones on the basis of our preparations, twin cones certainly predominate.

The dab and plaice (*Pleuronectes limanda* and *P. platessa*) may be considered together. The point of interest here is in the apparent lack of movement in the visual cells in response to light and dark adaptation. The material consisted of four dark adapted plaice eyes, two dark adapted dab eyes and three light adapted dab eyes. In all the dark adapted eyes the cones, if they had moved outwards at all, showed very little change from the light adapted condition. The main part of the rod cells was still definitely outside that of the cones. In the light adapted eyes the pigment had advanced inwards to form a dark opaque band between the outer part of the rods and the rest of the retina. Since none of the light adapted specimens was bleached it was very difficult to be certain about the presence of changes in the position of the rods but the general impression gained from a study of all these preparations was that the only change in the retinal picture as a result of changes in the adaptation was in the very obvious pigment movements. All the fish were in good condition with the exception of one dab which died after several hours' light adaptation. The pigment advance was quite normal in this animal. In this connexion it is well to remember the danger of drawing definite conclusions from the movements of the visual cells of a species from the study of a few specimens only. Walls, in a private communication, has pointed out the great individual variations that may occur. He found it necessary to average the results of ten or more animals in order to get reliable information. Also no attempt was made to keep the temperature constant during our experiments and although this probably did not vary more than a few degrees throughout the investigation it is well known that temperature has a marked effect on the visual cell movements.

Some twin cones were found in these retinae, thus confirming Krause's observation on another species of *Pleuronectes* (Krause speaks of double cones but probably is really referring to twin cones). Krause also remarks on the abundance of rods in the retina of *Pleuronectes* compared with the other fish he examined. In the two species examined here the unequal distribution of the rods and cones was very obvious, the cones being more numerous in the central area while the rods predominated at the periphery of the retina. Even in the light adapted specimens the

cones were much less closely packed than was usually observed in the teleost eye.

The finding of twin cones in the pollack agrees with the results of Friis (1879) and Krause (1886), who found mainly twin cones in various species of *Gadus*.

In general the results suggest that in the teleost retina at least the movements of the pigment and the visual cells in response to light may be the result of rather different mechanisms. Wunder (1924) found indications of the same thing. He observed in two fish (*Gobio fluviatilis* and *Gastereosteous aculeatus*) that by reducing the intensity of the stimulating light a point was reached at which the pigment could no longer respond although the visual cells still showed their normal movements.

Discussion

Throughout this paper the words "visual purple" have been used to denote the strongly light-sensitive substance which we have been estimating. We could not say with any conviction that the various forms described are confined exclusively to the rods as in frogs, mammals, etc. The histological evidence although suggestive is inconclusive. It is quite clear, however, that the light sensitive substances here described have the same general characters as has the substance generally known as visual purple. There is no positive evidence that visual purple plays any part in vision under high illumination, and if there is any explanation of our results it must be in terms of the low illuminations of twilight vision or, more topically, deep-sea vision.

The particular form of visual purple carried by a species might be determined by one of three factors:—(1) the depth at which it usually lives; (2) its ancestry, the original reason for the difference now being lost; and (3) an adaptation to the luminous emanations of its friends and foes. We are completely without evidence on the last point. We have made an attempt to find a connexion between the families of the fishes and the form of visual purple, taking as a basis Norman's "History of Fishes" (1931). The attempt was not successful since two fairly closely related genera such as the eel and the conger may have absorption maxima as widely separated as 505 $m\mu$ and 535 $m\mu$ respectively. The most probable explanation of the results would seem to be in terms of the depth or of the kind of water favoured by the species. Unfortunately there does not seem to be any very exhaustive work on the transmission of sea water. According to Sawyer (1931) a band of wave-lengths from 510 $m\mu$ to 540 $m\mu$ is transmitted equally well. If this is true then none of our fishes would be severely handicapped by going deep. A very

small error in the figure for the transmission will, however, be very serious in computing the transmission 100 metres down. If, however, we rely on Beebe's (1930) observations that a narrow band centred on 520 $m\mu$ is all that is left of the spectrum at 250 metres then the brightness of the light at this depth would vary from species to species, with the absorption of the visual purple at about 520 $m\mu$; those with a maximum absorption at 520 $m\mu$ would be expected to see better at extreme depths than those with maxima at 505 $m\mu$ or 545 $m\mu$. The data given in Table I are probably a good guide to the habits of our species down to depths of 300 metres. We are without information about distribution beyond this. There is no indication that the deep-water fishes possess visual purple specially suitable for use in light restricted to a wave-length of about 520 $m\mu$. Beebe (1934) however remarks that at 150 metres he saw species which are commonly regarded as surface forms, so that when more is known it may be possible to say that the plaice, wrasse, dragonet, and pollack frequent the great depths for which their visual purple suits them.

In Table I an approximation is given for the yield of visual purple per unit of retina. Owing to the waste involved in preparing clear

TABLE II

The absorption of light by visual purple at different wave-lengths. Absorptions are expressed as densities (D) where $D = \log_{10} \frac{I_0}{I}$. I_0 is the intensity of the incident light and I is the intensity of the emergent beam. The readings obtained have been multiplied by a factor to give a density of 1·0 at the wave-length of maximum absorption.

Wave-length $m\mu$	Dogfish	Eel	Mackerel	Dragonet	Ray	Plaice
440	0·492	0·552	—	0·189	0·169	0·040
460	0·742	0·734	0·490	0·440	0·447	0·290
480	0·947	0·942	0·772	0·744	0·739	0·635
490	0·997	0·957	0·902	0·873	0·882	0·775
495	0·986	0·982	0·961	0·934	0·938	—
500	0·999	0·993	0·987	0·976	0·968	0·895
505	1·000	0·999	0·999	0·982	0·995	—
510	0·974	0·967	0·962	1·000	0·999	0·960
515	0·942	0·935	0·917	0·984	0·985	0·995
520	0·877	0·881	0·870	0·948	0·945	1·000
530	0·768	0·676	0·697	0·846	0·860	0·942
540	0·610	0·652	0·521	0·700	0·678	0·850
560	0·354	—	0·223	0·372	0·340	0·530
580	0·225	—	0·068	0·165	0·133	0·272
600	0·152	0·216	0·003	0·051	0·051	0·073
650	0·096	0·160	−0·03	0·002	0·027	−0·003

TABLE II—(continued)

Wave-length $m\mu$	Wrasse	Pollack	Conger	Tench	Trout	Gurnard
480	0·370	0·324	—	0·467	0·328	0·216
490	0·555	0·435	—	0·631	—	0·241
500	0·766	0·568	0·243	0·820	0·655	0·364
510	0·936	0·741	0·479	0·872	0·796	0·497
515	0·952	0·825	0·627	—	0·875	—
520	0·983	0·909	0·760	0·950	0·932	0·714
525	0·999	0·934	0·880	0·971	0·944	0·795
530	0·962	0·997	0·968	0·978	0·952	0·898
535	0·929	0·998	1·000	0·997	0·997	0·939
540	0·929	0·954	0·947	0·995	1·000	0·982
545	—	0·898	0·854	0·982	0·982	0·998
550	0·891	0·843	0·719	0·955	0·945	0·973
560	0·772	0·619	0·467	0·917	0·884	0·791
570	—	0·427	0·274	0·830	0·760	0·524
580	0·439	0·257	0·172	0·738	0·626	0·345
600	0·195	0·084	0·073	0·450	0·402	0·104
650	0·026	0·013	0·053	0·046	0·031	0·020

solutions and uncertainty about the area of retina, quantitative values cannot be given.

We were unable to find any correlation between the type of visual cell predominating in the retina and the type of visual purple obtained. One might have expected to find a type of retina for instance adapted for taking its owner into dark places with a predominance of rods and a visual purple with the maximum adsorption at a relatively short wave-length. Alternatively, a retina with a good cone representation might produce a visual purple with a maximum absorption nearer the red end of the spectrum. It will be seen from Table II that of the two forms of rod retina (the elasmobranchs and conger eel) one had a maximum absorption of 505 $m\mu$ and the other at 535 $m\mu$.

Those fish which are generally supposed to use their eyes by day (mackerel, wrasse, pollack, and trout) certainly all have cones in the retina but so also have the plaice, dab, eel, and tench which are said not to do so. The tench particularly is known to go into deep water during the day while the eel only becomes active at night. Those fish which have a predominantly rod retina fit in much better with our general ideas. The ray, dogfish, and conger all become active at night. The dogfish is quite obviously nearly blind in a shallow tank in daylight but we have no data as to whether its eyes are any more useful in a dim light.

The conger eel is certainly capable of using its eyes at night and apparently does so more readily than during the day.

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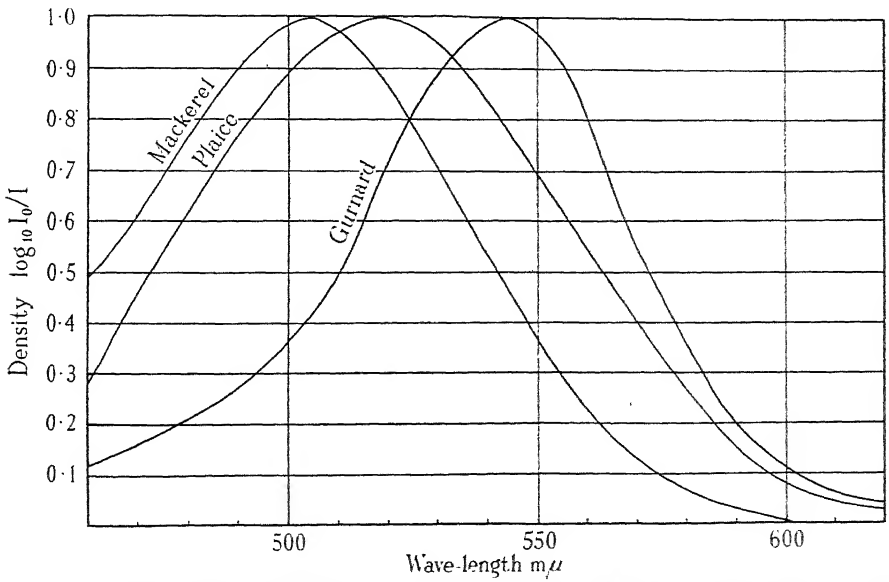

FIG. 3—Wave-length absorption curves for the visual purple of three species. The ordinates give the densities (D) of the solutions where $D = \log_{10} \frac{I_0}{I}$. I_0 = incident light. I = light transmitted. The actual readings are reduced to the same scale of ordinates.

SUMMARY

The paper describes a number of new forms of visual purple which are found in sea water fishes and which have maxima of absorption between 505 $m\mu$ and 545 $m\mu$.

The absorption curves were obtained by a null-point photoelectric spectro-photometer. The instrument is capable of giving accurate readings with 0·5 cc of solution and with a light intensity which is not sufficient to bleach the visual purple.

It was found that there are alterations in the form of the absorption curves as a result of "bleaching" of yellow substances in the control solution; also there may be changes in the curve when the visual purple is extracted with distilled water.

A histological examination of the retinae was made on each of the species used. The relative numbers and structure of the rods and cones; the movements of the visual cells as a result of light- and dark-adaptation; the movements of the pigment epithelium; and the staining reactions for visual purple are described.

The variety of visual purple carried by a species can be related neither to the available data on the depth which that species normally frequents nor to the ancestry of the species nor to the histology of the retina of origin.

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Description of Plates

Fig. 4—*Raia maculata*. × 300. Light adapted. F.W.A. and Mallory's triple connective tissue stain. Note the unpigmented epithelium.

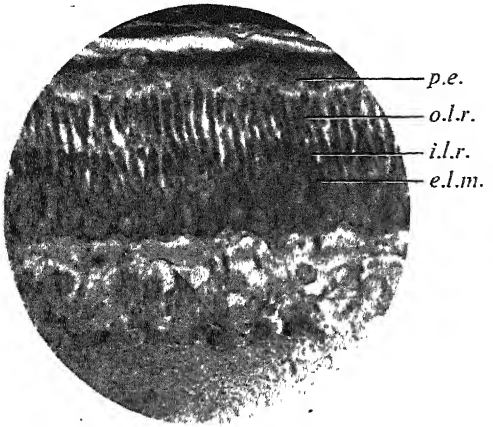

FIG. 4.

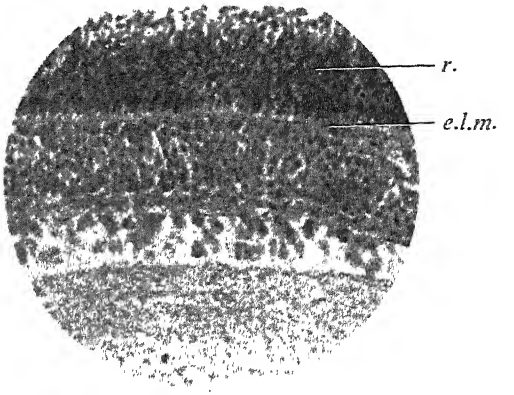

FIG. 5.

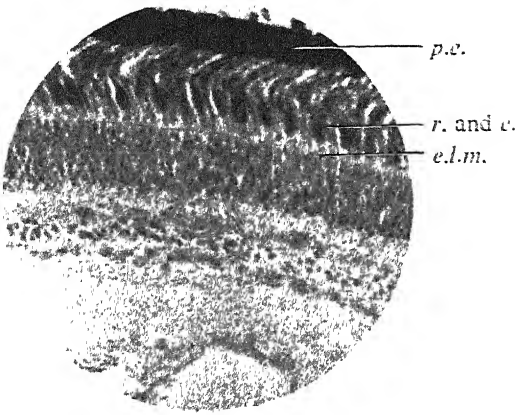

FIG. 6.

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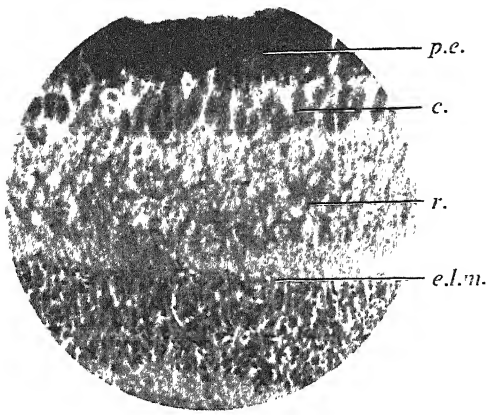

FIG. 7.

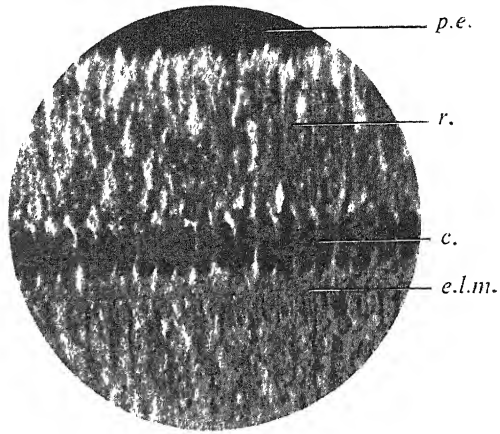

FIG. 8.

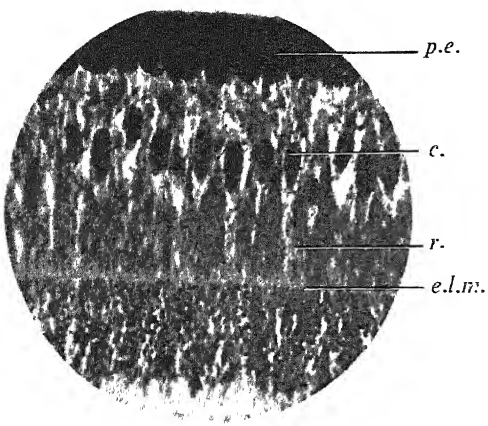

FIG. 9.

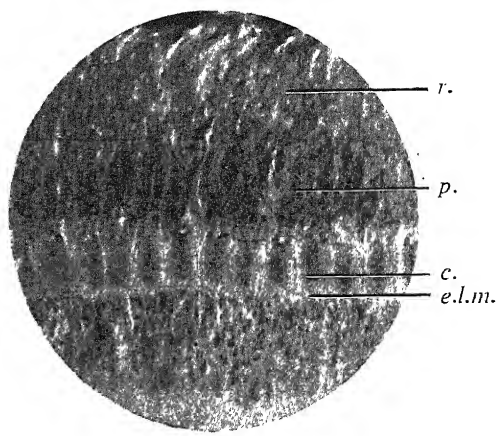

FIG. 10.

FIG. 5—*Conger vulgaris.* × 300. Dark adapted. F.W.A. and Mallory's phosphotungstic acid haematoxylin. The very fine and closely packed visual cells are probably rods.

FIG. 6—*Pleuronectes platessa.* × 300. Dark adapted. Zenker and Mallory's phosphotungstic acid haematoxylin. The cones have only advanced a short distance if at all.

FIG. 7—*Scomber scomber.* × 300. Dark adapted. Zenker and Mallory's phosphotungstic acid haematoxylin.

FIG. 8—*Scomber scomber.* × 300. Light adapted. Zenker and Mallory's phosphotungstic acid haematoxylin. The pigment has not advanced.

FIG. 9—*Gadus pollachius.* × 300. Dark adapted. Zenker and Mallory's phosphotungstic acid haematoxylin.

FIG. 10—*Gadus pollachius.* × 300. Light adapted. Zenker and Mallory's phosphotungstic acid haematoxylin.

p.e., pigment epithelium; *p.*, epithelial pigment; *o.l.r.*, outer limbs of rods; *i.l.r.* inner limbs of rods; *r.*, rods; *c.*, cones; *e.l.m.*, external limiting membrane.

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A Study of Anti-thyrotropic Activity

By I. W. Rowlands and A. S. Parkes, F.R.S.

(From the National Institute for Medical Research, Hampstead, London)

(*Received January* 22, 1936)

[Plate 6]

I—Introduction

The initial response of the thyroid gland to extracts of anterior pituitary containing the thyrotropic principle is intense hyperplasia of the vesicular epithelium with vacuolation and absorption of the colloid. In the guinea-pig the effect has been obtained with ease by many workers including Loeb and Bassett (1929, 1930), Aron (1929), Janssen and Loeser (1931), Junkmann and Schoeller (1932), and Anderson and Collip (1933). In the rat, the thyroid is normally somewhat hyperplastic, and the experimental production of increased hyperplasia seems to require concentrated thyrotropic preparations (Anderson and Collip, 1933). Coincidentally with the histological changes in the thyroid, general symptoms of hyperthyroidism appear; these include exophthalmos (Friedgood, 1934), and increase in the metabolic rate (Anderson and Collip, 1934, *a*). All effects of injection of thyrotropic hormone, however, are merely transitory and in spite of continued injections, the gland returns to normal and symptoms of hyperthyroidism disappear. Collip and Anderson (1934) find that the B.M.R. in rats returns to normal in two to three weeks and then drops to the level of that of the hypophysectomized rat, even if a large increase is made in the daily amount injected. Eitel and Loeser (1935, *a*) also secord a similar decrease to below normal in the B.M.R. of rats as the result of prolonged injection of thyrotropic extract. Schockaert (1932) for the duck, Hertz and Kranes (1934) for the rabbit, and Scowen and Spence (1935, 1936) for the guinea-pig, find that the hyperplasia produced in the thyroid in the first days of injections cannot be maintained and that involution, with regression of the epithelium, sets in.

The fact that prolonged treatment with thyrotropic extracts gradually becomes ineffective suggested that either (*a*) the thyroid itself becomes insensitive or exhausted, or (*b*) prolonged injection evokes the production of inhibitory or anti-thyrotropic substances. The latter view was shown to be correct by Collip and Anderson (1934), who found that the

serum of rats, after prolonged treatment, inhibited any change in the B.M.R. in hypophysectomized rats when given with as much as 200 times the minimal effective dose of the same extract. Collip (1934) also reported that a similar inhibitory serum could be produced by the prolonged injection of thyrotropic extracts into various species, including rabbit, goat, dog, and horse.

It is difficult to over-estimate the biological importance of this general conception of anti-hormones introduced by Collip and his co-workers. The essential facts have been confirmed by Eitel and Loeser (1935, *a*) who showed that the serum from a treated sheep possessed the power to inhibit the thyroid hyperplasia otherwise caused in guinea-pigs by the original thyrotropic extract. A similar experiment has been reported by Scowen and Spence (1935) using rabbit serum, and the results given in the present paper are a direct continuation of the work of Scowen and Spence and of that previously carried out by us (Rowlands and Parkes, 1934) on the assay of thyrotropic extracts. In view of the close correlation found between the weight of the thyroid of the immature guinea-pig and the dose of thyrotropic extract given, it seemed likely that it would be possible to assay any anti-thyrotropic power of blood serum by testing against a standardized thyrotropic extract.

II—Methods

Extracts—Two extracts, T17B and T18B, were prepared from acetone-desiccated ox pituitaries by extraction with 10 volumes N/20 NaOH, the extract being precipitated with 5 volumes 96% alcohol and 1 volume of ether. The precipitate when dried yielded about 10% of the original dry tissue. Four other extracts Pr.22, AP8B, AP14B, and AP15B, were made by treating the acetone desiccated ox pituitaries with 25 volumes of 50% aqueous pyridine, the extract being precipitated with alcohol and ether as above. Again the weight of the extracts obtained represented about 10% of the original dry tissue, or 2% of the fresh tissue.

Preparation of Animals—A group of adult rabbits (both sexes) was injected subcutaneously with 25 mg of extract daily. At various intervals, starting after 3½ weeks, 20 cc of blood was withdrawn from the marginal ear vein of each rabbit. The blood samples were mixed, allowed to clot at 37° C for one hour, and the serum collected. The serum was stored at −2° C and for short periods only.*

* In our experience the serum remains active for at least 6 weeks under these conditions, but Collip and Anderson (1935) found that their serum lost its potency when kept sterile in cold storage for 2 months.

Immature female guinea-pigs weighing about 200 gm were used for testing serum. They were injected subcutaneously daily for 5 days with standardized thyrotropic extract and with the serum The two preparations given to the same animal were injected independently, one immediately following the other. The animals were killed and weighed 24 hours after the last injection. The thyroids were dissected out, fixed in aqueous Bouin's fluid overnight, and weighed on a torsion balance, from 70% alcohol. Most of the thyroids were stored in alcohol, but those prepared for histological examination were embedded, cut at 7 μ and stained in haemalum and eosin.

Ten guinea-pigs were used in each group test. The criterion of response was taken, in all cases, as the average increase in weight of the thyroid above the normal for the same body weight, since some slight variation in the average body weight of the groups was inevitable. The normal value was calculated from the linear regression given by Rowlands and Parkes (1934), $y = 0 \cdot 159x - 1 \cdot 02$, where y = thyroid weight and x = body weight. The guinea-pigs were from the same stock as those previously employed by us so that variation in the thyroid was reduced so far as possible. The technique depends entirely on the availability of large numbers of healthy young guinea-pigs of uniform stock and rearing, and is made possible for us only by the Institute's large breeding colony.

Other details of technique are given in the appropriate places.

III—Comparison of the Potency of the Extracts

Table I gives the increase in weight of the thyroids (above normal for the same body weight) caused by the injection of varying amounts of different extracts.

Table I—Activity of Various Thyrotropic Extracts Tested on Immature Guinea-pigs

Amount of extract given, mg daily	Increase in weight of thyroid above normal mg					
	T17B	T18B	Pr.22	AP8B	AP14B	AP15B
1·5	—	—	—	21·9	—	—
2	21·3	—	23·2	—	—	27·0
3	—	—	—	37·4	—	—
4	32·0	—	45·0	41·5	35·0	37·0
5	(31·4)	34·4	—	—	—	—
7	49·0	—	59·5	51·0	—	54·0
10	49·0	—	—	—	—	—

The similarity in potency of the three pyridine extracts for which there are full data, is shown in fig. 1. The unit adopted by Rowlands and Parkes (1934) in the assay of thyrotropic extracts on 200 gm female guinea-pigs is defined as that weight of extract which will cause the thyroids to double their weight. The normal thyroid weight of the guinea-pigs used is about 30 mg, so that 1 unit represents a 30 mg increase, which means that the weights of the units for the three extracts Pr.22, AP8B, and AP15B fall between 2·25 and 2·75 mg. The alkali extracts, T17B, T18B, and the pyridine extract AP14B, appear to be slightly less active.

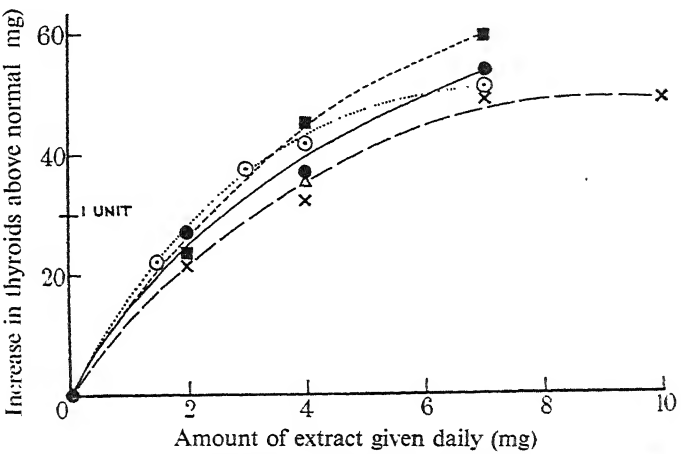

FIG. 1—Dose response curves for various thyrotropic extracts. ● — AP15B; ···⊙ AP8B; Δ, AP14B; - - - ■ PR22; — — × T17B.

IV—THE ALLEGED ANTI-THYROTROPIC ACTIVITY OF NORMAL BLOOD

In this study of the production of anti-thyrotropic activity in the blood of animals treated with thyrotropic extracts of pituitary it was essential to find out to what extent, if any, such activity is possessed by normal blood. Eitel and Loeser (1935, *a*) found that it was present in normal sheep serum, but that if the sheep had been previously treated with thyrotropic substance the inhibitory effect of the serum was considerably enhanced. They state (Eitel and Loeser, 1935, *b*) that 2 cc of normal sheep serum given daily for 6 days inhibited the action of 20 guinea-pig units of thyrotropic substance. Herold (1934) also noted that normal guinea-pig serum inhibited the effect of thyrotropic extract. In contrast to these positive results with normal serum, Anderson and Collip (1934, *a*) found that normal rat serum was incapable of preventing the increase in the basal metabolic rate of rats treated with thyrotropic hormone. They

have similarly shown (Anderson and Collip, 1934, *b*) that normal horse serum has no anti-thyrotropic activity. In view of these divergent results, the normal serum of a number of species was examined. One cc of serum of the various species (*see* Table II) was given subcutaneously with 4 mg of thyrotropic substance.

TABLE II—ANTI-THYROTROPIC ACTIVITY OF THE SERUM OF VARIOUS SPECIES TESTED AGAINST CONSTANT DOSE OF THYROTROPIC EXTRACT

Species—serum used	Body weight of test guinea-pigs gm	Thyroid weight of test guinea-pigs mg	Increase of thyroid weight over normal mg
Horse	203	65	34
Rabbit	199	75	44
Cow	234	87	51
Goat	177	72	45
Sheep	177	65	38
Thyrotropic extract only (Pr22) 4 mg	187	74	45

Of the sera tested, that of the cow showed no inhibitory activity; the thyroids of the test guinea-pigs were actually slightly heavier than those of the control animals given thyrotropic extract only. No anti-thyrotropic activity was found in either the rabbit or the goat serum, but a slight inhibition was apparently given by that of the sheep and the horse.

V—THE EXPERIMENTAL PRODUCTION OF ANTI-THYROTROPIC ACTIVITY IN BLOOD

With these results on the alleged anti-thyrotropic power of normal serum before us, we proceeded to test the serum of animals injected for a long period with thyrotropic extract.

In the first experiment, daily injections for 4 weeks of 100 mg of thyrotropic extract (T18B) were given to a large castrated wether. At the end of each week the serum was tested by injecting it, together with the original extract, into guinea-pigs. The serum after 4 weeks of injection showed no significant anti-thyrotropic power. It will be seen, however, in comparison with later results, that no great inhibition is to be expected by this time, although Eitel and Loeser (1935, *a*) found by similar treatment of a 40 kg sheep that the anti-thyrotropic power of the serum increased to a maximum at 4–5 weeks, and then declined.

In the next experiment, rabbits were injected daily for 18 weeks with 25 mg of thyrotropic extract (AP8B). During the last 8 weeks, serum was obtained weekly and tested against the same thyrotropic extract on immature guinea-pigs. The dose response curve given by the extract alone is shown in fig. 1, and as the top line in fig. 2. The same amounts of extract, given first in conjunction with 1 cc daily of the serum and then with 2 cc daily, produced the results shown in the two lower lines in fig. 2 (actual data in Table III). From this diagram it will be seen that 1 cc of the serum completely inhibits 2 mg of the extract, reduces the activity of 4 mg to about one-half, and inhibits about one-fifth of the activity produced by 7 mg. 2 cc of serum, on the other hand, completely

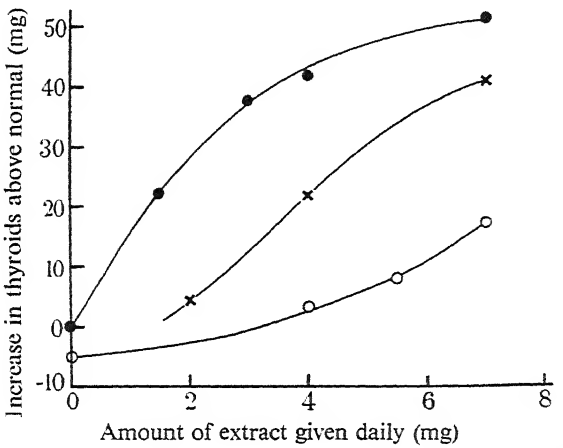

FIG. 2—Effect of anti-thyrotropic serum on dose response curve for thyrotropic extract. ● Thyrotropic extract only (AP8B); × extract + 1 cc serum; ○ extract + 2 cc serum.

inhibits 4 mg and reduces the activity of 7 mg to about one-third. 2 cc of this serum alone depresses the weight of the thyroid to slightly below normal.

The histological appearance of these thyroids fully substantiated the indications of the weight changes. Fig. 5, Plate 6, shows a peripheral area of the normal thyroid of the guinea-pig, this part being less active than the centre of the gland (Heyl and Laqueur, 1935). In this region the vesicular epithelium is inactive, consisting of small flattened cells, with little cytoplasm; the lumen contains dense, unvacuolated colloid. Stimulation of the thyroid by thyrotropic extracts results in a uniform hyperplasia of the gland. Fig. 6, Plate 6, is taken from an area comparable to that shown in fig. 5, and shows the result of stimulation of the thyroid by 4 mg of thyrotropic extract over 5 days. The highly vacuolated

colloid is being actively absorbed and the epithelial cells are greatly hypertrophied. When 2 cc of the serum is given in conjunction with the extract, no such stimulation is obtained, fig. 7, Plate 6, inhibition is complete and the gland appears quite normal.

We considered these experiments to be a convincing demonstration of the presence of anti-thyrotropic activity in the serum of long-treated animals. We next investigated the rate of appearance of this activity.

III—Anti-thyrotropic Activity of the Serum of Rabbits after Prolonged Injection of Thyrotropic Extract

Amount of thyrotropic extract (mg) given to test guinea-pigs	Body weight of test guinea-pigs gm	Amount of rabbit serum cc	Thyroid weight of test guinea-pigs (mg)	
			Actual	Increase over normal
1·5	197	—	52·2	21·9
3·0	138	—	58·3	37·4
4·0	186	—	70·0	41·5
7·0	181	—	78·8	51·0
2·0	206	1	36·0	4·3
4·0	190	1	50·5	21·3
7·0	190	1	69·8	40·6
0·0	194	2	25·0	−4·8
4·0	187	2	31·7	3·0
5·5	197	2	38·0	7·7
7·0	187	2	28·7	17·0

Again a group of rabbits was treated daily with 25 mg of thyrotropic substance (AP14B). After 3½, 5, 7, and 10 weeks, 2 cc of the serum together with 4 mg of the thyrotropic extract was injected daily into guinea-pigs, the results being shown in Table IV and fig. 3. The latter gives the amount of thyrotropic extract inhibited by 2 cc of the serum at the given times, these amounts being calculated from the curve given for AP14B in fig. 1.

This experiment shows that anti-thyrotropic activity develops rapidly in the blood after 4 weeks, and reaches a maximum at 10 weeks. Assuming that the rate of production in the two species is similar, the fact that the sheep was injected for only 4 weeks would explain the failure of its serum to inhibit appreciable amounts of thyrotropic substance.

After 10 weeks of injections, there was no obvious decrease in anti-thyrotropic activity, such as Eitel and Loeser found, even after injections

TABLE IV—APPEARANCE OF ANTI-THYROTROPIC ACTIVITY IN SERUM OF RABBITS INJECTED WITH THYROTROPIC EXTRACT

Period in weeks after first injection when serum obtained	Body weight of test guinea-pigs when killed gm	Weight of thyroids of test guinea-pigs (mg)		Amount of thyrotropic extract inhibited mg
		Actual	Increase above normal	
*—	161	59·6	35	0
3½	203	61·7	30·5	0·8
5	191	44·1	14·8	2·8
7	210	40·8	7·4	3·4
10	193	29·4	−0·3	4·0

Each animal received 4 mg AP14B + 2 cc of serum daily.
* 4 mg given alone, normal serum previously found to be inactive.

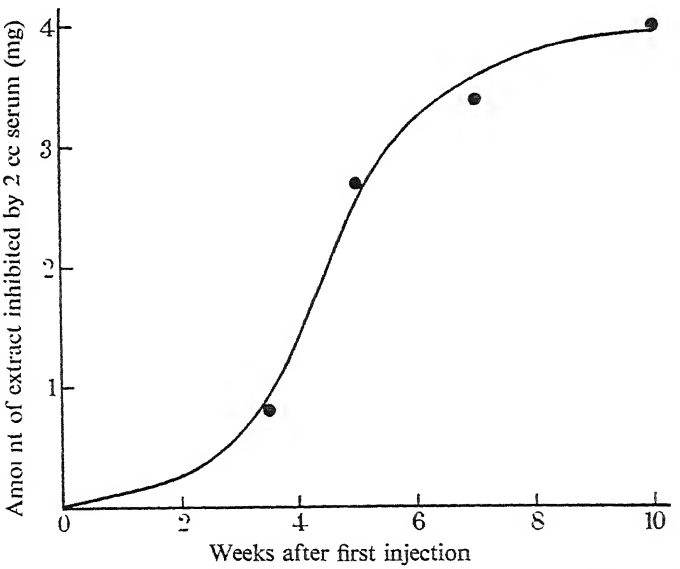

FIG. 3—Development of anti-thyrotropic activity in serum of rabbits injected with thyrotropic extract.

were continued up to 18 weeks. Collip and Anderson (1934) found that the serum of rats remained highly anti-thyrotropic from 6 to 8 weeks.

VI—STANDARDIZATION CURVE FOR ANTI-THYROTROPIC SUBSTANCE

The data for the standardization curve shown in fig. 4 were obtained by giving 4 mg of AP15B to guinea-pigs in conjunction with varying amounts of the anti-thyrotropic serum, viz., 0, 0·5, 1, 1·5, and 2 cc

daily. A group of ten animals was used for each test. It can be seen that 0·5 cc of serum neutralizes about one-half the activity contained in 4 mg of the extract, and that 2 cc completely inhibits that amount. In comparison with the results shown in fig. 2, the serum appears more able to inhibit AP15B than AP8B. When 1 cc of serum is given with the latter, the increase in weight of thyroids is 23 mg while with AP15B it is only 9 mg.

TABLE V—DOSE/RESPONSE RELATION FOR ANTI-THYROTROPIC ACTIVITY

Amount of serum daily cc	Body weight of test guinea-pigs gm	Weight of thyroids of test guinea-pigs (mg)	
		Actual	Increase above normal
—	195	67	37
0·5	182	49	21
1	188	38	9
1·5	192	33	3
2	195	30·5	0·5

All groups received 4 mg of AP15B daily.

By plotting the individual weights of the thyroids obtained in these five groups it was seen that the gross variability remained approximately constant throughout the groups. In view of this, a standard deviation was calculated for all the 50 observations together, giving $\sigma = 6 \cdot 9$. The standard error, therefore, of any particular mean response in a group of 10 animals is $\pm 2 \cdot 3$, and the error in the determination of the amount of anti-thyrotropic activity is about $\pm 12\%$.

The comparative constancy of the variability in the groups is of interest, particularly since the same was found for the variability of the direct response of the thyroid to thyrotropic extracts. Contrary to this finding, Deanesly (1936) observed that the variability in the response of the immature rat ovary to the gonadotropic substance increased with the degree of response.

At the present time no good purpose can be served by trying to define a unit of anti-thyrotropic activity.

VII—DISCUSSION

These experiments show quite definitely that prolonged treatment with thyrotropic hormone induces anti-thyrotropic properties in the blood of rabbits, which becomes capable of inhibiting the activity of the original

extract on the thyroid of test animals. So far we have entirely confirmed the essential feature of the anti-hormone conception of Collip and his co-workers. Many obvious problems arise at this stage. Some of the chief are:—

(*a*) Is the acquisition of the anti-thyrotropic power due to the formation of an antibody in the immunological sense of the word?

(*b*) Is the acquired immunity specific for the extract evoking it, *i.e.*, would the serum inhibit (1) extracts of pituitaries of different species and (2) extracts differently prepared?

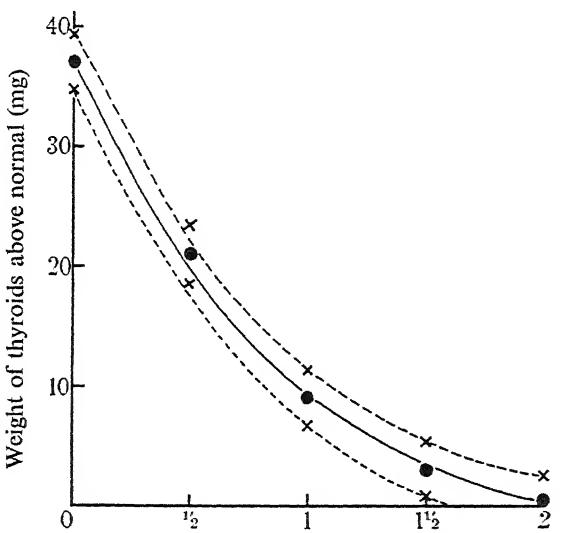

FIG. 4—Dose response curve for anti-thyrotropic activity. ● mean values observed; × standard error.

(*c*) As an extension of (*b*), will the serum transferred to another animal protect that animal against its own pituitary hormone and lead to changes similar to those following hypophysectomy? On the answer to this question will mainly depend the clinical value of anti-hormones.

(*d*) Does the animal produce any anti-body to its own pituitary hormones, *i.e.*, is the functional activity of, say, the thyroid, the expression of a balance between thyrotropic and anti-thyrotropic substance (Collip, 1934), or is anti-thyrotropic activity merely an artifact produced by the introduction of foreign protein into an animal?

(*e*) What are the chemical and other properties of the anti-thyrotropic substance, what possibilities are there of purifying serum fractions or even of isolating the substance, and can stable preparations be made?

Some of these and similar questions have been partially answered by Collip and others, but in spite of their pioneer work, very much remains to be elucidated.

We would express our indebtedness to Sir P. P. Laidlaw, F.R.S., and Professor C. R. Harington, F.R.S., who have very kindly undertaken work on the immunological and chemical aspects respectively; to Dr. A. W. Spence and Dr. E. F. Scowen, at whose suggestion the rabbit experiments were begun; and to Professor J. B. Collip, F.R.S., who read the manuscript of the present paper.

Summary

Inhibition of the effect of thyrotropic extract on the weight of the thyroid of the immature guinea-pig (Rowlands and Parkes, 1934) has been used as a test for anti-thyrotropic activity.

The normal blood serum of the goat, horse, sheep, cow, and rabbit was not found to possess appreciable anti-thyrotropic activity, nor was that of a castrated wether injected with thyrotropic extract for 4 weeks.

Anti-thyrotropic activity was induced in the blood of rabbits injected daily with thyrotropic extract over a long period. The activity began to appear after 4 weeks' injection, and rose to a maximum at 10 weeks. 2 cc of serum obtained at this time completely inhibited the activity of an amount of thyrotropic extract otherwise sufficient to double the weight of the thyroids of immature guinea-pigs.

A technique is described for the assay of anti-thyrotropic activity.

The results described are in complete agreement with the main features of the anti-hormone conception of Collip and his co-workers.

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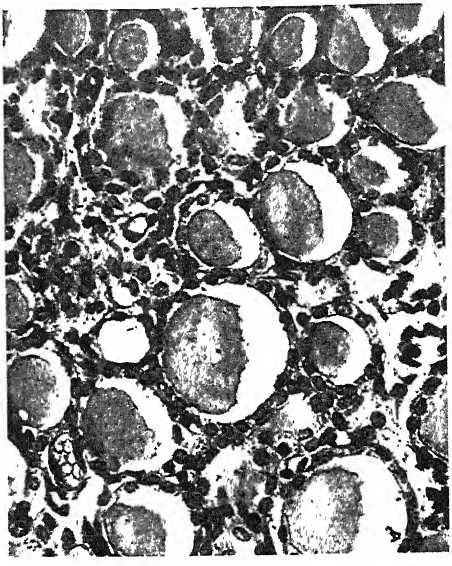

FIG. 5.

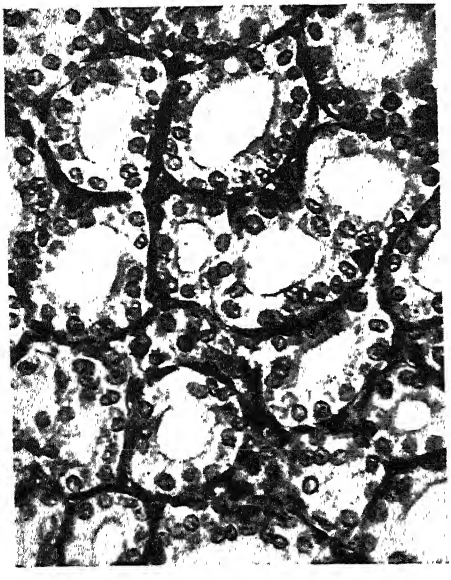

FIG. 6.

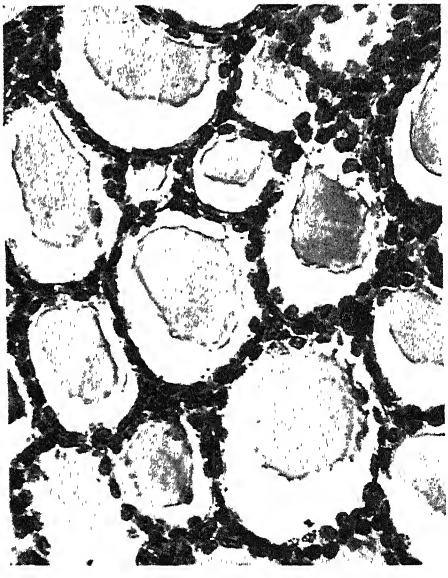

FIG. 7.

(*Facing p.* 125)

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Description of Plate 6

Fig. 5—Thyroid of normal immature guinea-pig, showing accumulation of colloid and inactive vesicular epithelium. × 307.

Fig. 6—Thyroid of guinea-pig injected with 4 mg thyrotropic extract for 5 days showing hyperplasia of the gland. The vesicular epithelium is very active, and the colloid highly vacuolated. × 307.

Fig. 7—Thyroid of guinea-pig given 4 mg of the extract daily for 5 days, but receiving, in addition, 2 cc anti-thyrotropic serum daily. The appearance is that of a normal thyroid. × 307.

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Studies in the Geotropism of Pteridophyta*
VI—On Rhythm in Graviperception and Reaction to Gravity

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I—INTRODUCTION

Recurrent phenomena in living things such as the yearly fall of the leaf, or the daily opening and closing of certain flowers, is a matter of common observation. Many other less obvious cases have been disclosed in biological research; such as the annual opening and closing of lenticels, and the diurnal periodicity of secondary thickening (Kaiser, 1879) and of osmotic pressure (Ursprung and Blum, 1916); but so far as I am aware, annual rhythm has not been demonstrated for any form of irritability, and neither annual nor diurnal rhythm for any tropism. The present work attempts to show that the perception of, and response to, gravity are not of the same intensity at all periods, but vary both with the time of day and season of the year. During the course of a series of researches on geotropism in the Pteridophyta the first of which was communicated to the Royal Society in 1921 (Prankerd, 1922) the impression was gained that the plants were less responsive during the winter, and it was decided to investigate the matter quantitatively for the fern whose behaviour to gravity was best known, namely *Asplenium bulbiferum*. The work was performed in daylight, partly because a dark room at constant temperature for long periods of time was impracticable, but more especially because it was desired to keep the plants under perfectly natural conditions, and thoroughly healthy. That they were so was shown by their general appearance, growth, and reproduction. Precautions against heliotropic curvature were taken as fully explained previously (Prankerd, 1922). The temperature of the research greenhouse in which the plants were grown was usually near 20° C, and all experiments recorded in this paper were performed at 20° ± 1°, and at a relative humidity of about 80%. Hence, with the exception of a some-

* Previous studies in this series are cited at the end of this paper.

what lower average temperature at night, and of course less daylight in the winter, every external condition to which the plants were subjected would seem to have been the same throughout the year, and their behaviour as far as could be seen, for example, in growth and reproduction, was fairly uniform.

Previous work has shown that the irritability of the frond to gravity varies with its development (Waight, 1923). It is very low in the early stages, requiring long periods of stimulation, and as response is also slow, experiments are very lengthy. In its later stages the frond is somewhat uncertain in its behaviour, and more sensitive to light stimulus. The middle stages, *i.e.*, "late infant" and early "adolescent" (*see* below), have therefore always been employed. They have been more fully studied, are the most reactive, and are more constant, at least than the later stages.

The stimulus was given by placing the young frond horizontally, and ended by replacing it upright after a measured period of time. This method has the practical advantage of enabling fairly large numbers (a dozen or even twenty) of experiments to be made concurrently and therefore under more closely similar conditions. A white card was placed beneath the frond to reflect the light on to its lower side, and when necessary it was supported by a small pad of cotton wool. The period of stimulation was always (except in the work on increased stimulus, *see* § III, p. 132) the presentation time for the particular stage used, already worked out by my student Miss Waight (1923). Presentation time was then defined as that period "which under the given conditions will generally produce movements of about 5° and only very rarely exceeding 10°".

Although not stated, the impression given was that about four-fifths of the fronds responded to the presentation time in the experiments cited, which were mostly performed in the summer. My experiments in summer months gave exactly 80% response, so that a more precise definition of presentation time as applying to this work can now be given, *i.e.*, *the minimum period of stimulation under the given conditions which will produce movements of about* 5° (*or only exceptionally over* 10°) *in approximately* 80% *of the fronds used.*

The following abbreviations are employed in this paper:—

L.I. = late infant stage, *i.e.*, leaflets still enclosed in the apical coil, but the first pair visible and directed more or less downwards.

A1, 2, 3, etc. = adolescent 1, 2, 3, etc., *i.e.*, 1st, 2nd, 3rd, etc., pairs of leaflets expanding and flush with the apical coil.

L.T. = latent time, *i.e.*, " the period elapsing between the beginning of stimulation and the first indication of response " (Prankerd, 1922, p. 146).

P.S. = period of stimulation, and P.T. = presentation time.

The actual values in hours of the P.T. and L.T. for the stages used (here referred to as " standard " time) are:—

Stage	P.T.	L.T.
L.I.	2·0	6·0
A1	1·5	5·5
A2	1·0	5·5
A3–4	0·75	5·5
A5–7	0·5	5·0
A8	1·0	6·0

(A5–7 were not often used, and A8 very rarely.)

In the tables the stage and length of the frond are usually stated, as giving some idea of its morphological development; and the growth per day is also entered as perhaps the best available and most easily measurable quantity indicating its physiological activity.

II—The Annual Rhythm of *Asplenium bulbiferum*

Preliminary series of experiments made in January and February under conditions of temperature and humidity similar to those of the summer, indicated that a marked increase in sensitivity took place in the first fortnight of February as compared with the last fortnight of January. This result was confirmed by further series carried out during the winter. In order to gain some measure of this fluctuation, the method was tried of attempting to increase the period of stimulation until the same results occurred as in the summer, and so of expressing the decrease in irritability as some multiple of the summer presentation time. As the work proceeded it became evident that such a period did not exist (*see* § III, p. 132). The method was therefore employed of using the same stimulus, *i.e.*, the summer P.T., and estimating the perceptive power by the number of fronds responding. As large numbers as possible were used in order to minimize the effect of individual variation, which as the tables will show is considerable. It was further thought that the suspected periodicity was worth investigating throughout the year, and would be most completely exhibited by means of experiments carried out in consecutive

months. These were accordingly begun in October, 1924, and continued each month (except February, 1925) till June, 1926. In all over 650 experiments were made, nearly half of which were in the winter. A few series illustrative respectively of different seasons of the year will first be given, in Table I, and then the average results of all experiments in Table II. It should be noted that the angle of curvature shown is always the greatest attained. Thus the A3 frond in Table I (August, 1925) was estimated to have moved through 3° at the end of 5 hours, and through 12° more in a further 1½ hours. Hence 15° is entered under the angle curvature of column, and 5 hours under the L.T. column.

TABLE I

	Stage of frond	Length cm	Growth increment day cm	Angle of curvature	L.T. hours
				°	
August 5, 1925	A1	5·25	1·2	25	4·0
	A1	10·0	1·1	18	5·0
	A3	10·2	2·0	15	5·0
	L.I.	6·3	0·8	5	6·0
	L.I.	5·9	0·8	5	7·5
	A1	5·3	0·9	5	7·0
	A1	4·8	1·1	5	5·0
	L.I.	9·2	1·1	3	8·5
	L.I.	5·3	1·3	3	7·5
	L.I.	6·2	0·3	3	9·0
	A3–4	4·8	0·6	3	4·5
	L.I.	3·5	0·6	3	7·0
	A2	5·5	0·5	—	—
	A3–4	5·7	1·3	—	—
	A5	5·5	0·5	—	—
November 17, 1924	A3	11·9	1·1	30	5·5
	A1	7·65	0·85	8	11·0
	A2	7·2	0·7	5	10·0
	A8–9	17·5	1·4	5	8·0
	A3	15·3	1·4	5	5·5
	A5	9·9	0·4	5	6·0
	A1	9·1	1·0	—	—
	L.I.	8·55	0·85	—	—
	A1	5·7	0·6	—	—
	A1	3·85	0·45	—	—
January 19 and 22, 1925 ..	A1	5·7	0·8	6	6·5
	A1	5·4	0·7	6	6·5
	A2	8·1	1·2	5	6·5
	A2	13·5	0·7	5	7·0

TABLE I—continued

	Stage of frond	Length cm	Growth increment /day cm	Angle of curvature °	L.T. hours
January 19 and 22, 1925 ..	L.I.	5·85	0·55	5	7·0
	A2	7·0	0·7	5	12·0
	A5	12·5	1·5	4	5·25
	L.I.	5·1	1·1	4	7·0
	A4	18·0	2·3	—	—
	A5	14·25	1·05	—	—
	L.I.	5·1	0·9	—	—
	L.I.	4·0	0·6	—	—
	L.I.	5·75	0·25	—	—
February 4, 1926	A1–2	11·5	1·1	5	5·5
	A1	6·8	1·0	5	6·0
	A7	18·4	1·3	5	7·0
	A1–2	8·0	0·8	5	7·0
	L.I.	6·5	0·8	5	7·0
	A1	5·2	1·1	5	7·5
	A1	4·7	0·9	3	7·5
	L.I.	4·1	0·9	3	7·5
	A6	11·6	0·9	—	—
	A2–3	6·8	0·8	—	—
	L.I.	4·25	0·35	—	—

The series of August 5, 1925 (above) is fairly typical of nearly 500 experiments carried out in the summer months of 1922, 1925, and 1926. In the November series the number responding has dropped, while the rise in the L.T. is even more marked. January gives a very similar series showing that winter conditions still prevail, but in February sensitivity rises with a corresponding fall in the L.T. The average results of all experiments made in 1924–26 are shown for each month in Table II, where the L.T. is expressed as the average percentage increase in the L.T. previously established (= "standard" L.T., *see* p. 128). This is more accurate than the average L.T., since the latter depends in part on the stages of the actual fronds used. Fortunately figures were also available for January, 1924, hence we have for comparison data relating to three winters as well as two summers.

From Table II it can be seen that the months yielding the highest number of responding fronds with the lowest increase in the L.T. are May to September. These therefore constitute the summer months, and the results are in very close accord with those already published (Waight, 1923), *i.e.*, an average angle of 6° is moved through in about

5 or 6 hours by a majority of the fronds. Waight's work was based on some 300 experiments, and these are now confirmed by another 170 establishing the proportion responding as 80%. Turning to those months which gave the lowest percentage of response, we find that they are those

TABLE II

Date	No. of experiments	Average growth increment day cm	Percentage response	Average angle of curvature °	Average percentage advance on "standard" L.T.
1924					
January	19	0·6	63	7·0	29·0
October	22	1·0	82	5·2	1·6
November	30	0·8	73	8·0	36·6
December	32	0·8	66	5·0	32·5
1925					
January	56	1·0	61	7·3	38·0
March	13	1·0	77	6·8	24·0
April	17	1·1	70	6·0	11·0
May	25	1·4	76	5·2	13·0*
June	31	1·0	81	4·9	13·5*
July	30	0·9	77	5·8	6·6
August	35	1·1	86	6·9	1·2
September	25	1·0	80	5·0	0·0
October	31	1·1	74	4·8	18·0
November	45	0·9	62	5·1	18·0
December	41	0·8	63	8·1	30·0
1926					
January	64	0·9	66	5·8	32·0
February	47	1·0	68	6·2	23·0
March	32	0·9	78	5·4	11·0
April	34	1·2	79	5·3	−5·5
May	16	1·0	81	6·0	0·0
June	11	1·0	82	9·0	−12·0

* This high value (*cf.* May and June, 1926) is accounted for by the fact that some of the experiments were made on hot days, when the temperature was only kept down to 20° C by allowing a draught through the greenhouse. The humidity was thus lowered with a consequent rise in the L.T. Work is contemplated with a view to ascertaining the exact effect of humidity on L.T.

with the highest increase in the L.T., *i.e.*, November to January. These are therefore considered the winter months for geotropism in this particular plant, leaving February to April with figures approximately half-way for its spring, and the single month of October as its autumn. Table III

gives the seasonal averages compiled from Table II in accordance with the above divisions, and the results are expressed graphically in fig. 1. The "perception" curve is plotted from the percentage of fronds responding, the curve for "response" from the L.T. column, while the curves marked "angle" and "growth" give respectively the corresponding average angle of curvature and average growth increment per day.

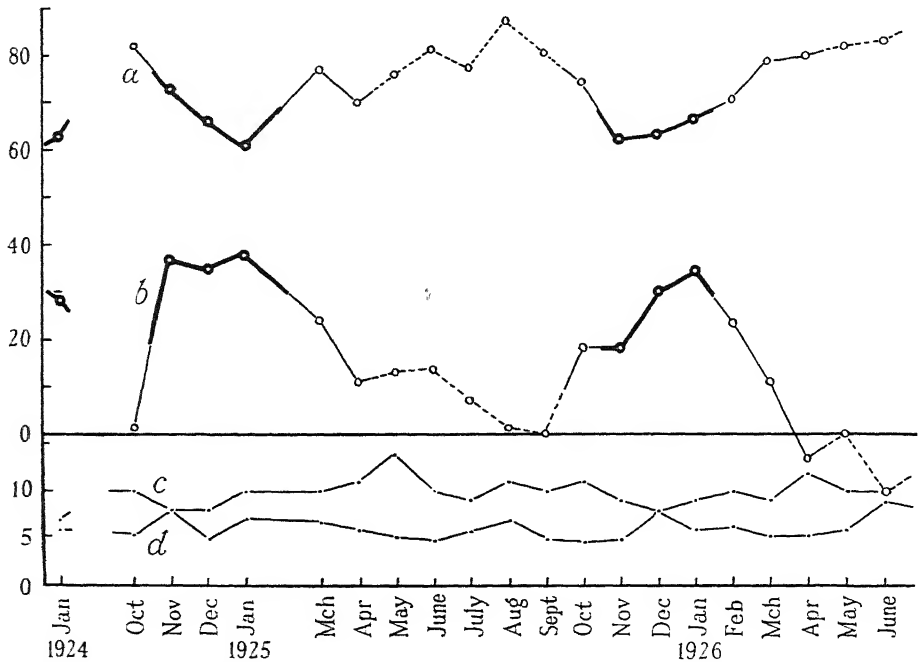

FIG. 1—Graphical expression of yearly rhythm in fronds of *Asplenium bulbiferum*. *a*, perception; *b*, response; *c*, growth; *d*, angle.

It is thus demonstrated that whereas the season of the year has no effect upon the amplitude of movement there is a fall in the winter of 20% in the perceptive power, and of 30% in the quickness of response.

III—The Effect of Stimuli above the Threshold of Stimulation

Over 120 experiments were performed in which the P.S. was some multiple of the P.T. As previously stated (p. 128) this was originally done in order to express the winter decrease in sensitivity in terms of the P.T. Although perhaps insufficient for conclusive results, the figures may at least afford indications of value; particularly as previous workers

(Bach, 1907) have employed almost exclusively centrifugal force, which unlike gravity is not a normal condition of plant life.

Table IV sets forth the experiments made in August and September in which a stimulus of 3 × P.T. was used, and Table V gives a similar series carried out in February. The last horizontal line gives the average quantities.

TABLE III

Season	No. of experiments	Average growth increment day cm	Percentage response	Average angle of curvature °	Average percentage advance on "standard" L.T.
Spring (Feb.–April)	151	1·0	75	5·9	12·3
Summer (May–Sept.)	173	1·1	80	6·0	1·4*
Autumn (Oct.)	53	1·0	77	5·0	11·2
Winter (Nov.–Jan.)	287	0·9	64	6·5	32·0

* Neglecting May and June, 1925.

TABLE IV—AUGUST AND SEPTEMBER

Stage of frond	Length cm	Growth increment day cm	Angle of curvature °	L.T. hours
A7	12·3	1·8	9	5·0
A6	11·6	0·7	10	5·0
A1	5·0	1·0	15	6·0
A1	6·1	0·9	17	5·5
A1	6·1	0·7	20	6·0
A1	6·7	1·6	21	4·0
		1·1	17	1% advance on "standard" L.T.

TABLE V—FEBRUARY

Stage of frond	Length cm	Growth increment day cm	Angle of curvature °	L.T. hours
A4	12·0	0·7	5	9·0
A3	9·4	0·8	10	6·5
A2	10·8	0·8	20	5·0
A5	18·6	1·8	20	5·0
		1·0	13·8	19% advance on "standard" L.T.

Even these figures, in spite of the great individual variation, clearly indicate that it is the amplitude of movement and not the time of reaction that is seriously affected. In some January experiments recorded in Table VI a stimulus of 1·5 P.T. was used, and here again it is the angle of curvature which has risen, though the L.T. is actually lower than is normal for this month.

TABLE VI

Stage of frond	Length cm	Growth increment/day cm	Angle of curvature °	L.T. hours
A1	5·4	0·9	5	6·5
A3	8·4	1·4	5	5·0
A3	8·6	0·8	6	5·5
A3	6·9	1·4	8	8·0
A2–3	8·15	1·9	8	6·0
L.I.	4·1	0·9	8	8·0
L.I.	6·7	1·1	8	6·0
A6	20·5	1·5	10	9·0
A1	7·8	1·7	12	6·0
A7	10·9	1·1	—	—
A5	8·5	0·7	—	—
A1	5·6	1·2	—	—
		1·2	7·8	20% advance on "standard" L.T.

A wider basis for comparison is given in Table VII which records the total results for all experiments performed with increased stimuli in January and February, 1926. Although strictly January is a winter month and February a spring month for our plant, it will be seen from Table II that the results for perception when 1 × P.T. was used did not substantially differ in this year.

TABLE VII

P.S.	No. of experiments	Average growth increment/day cm	Percentage response	Average angle of curvature °	Average percentage advance on "standard" L.T.
P.T.	111	0·9	67	6·0	30
1·25 × P.T.	34	1·0	68	7·9	22
1·5 × P.T.	37	1·0	81	7·5	26
2·0 × P.T.	32	1·0	84	10·3	17
3·0 × P.T.	19	1·1	95	13·1	18

It seems evident that since 3 × P.T. does not lower the L.T. below its value for 2 × P.T., this quantity cannot be reduced in the winter to its summer level. Hence although 1·5 × P.T. in the winter gives about the same percentage response as 1 × P.T. in the summer, it cannot be said to produce the same results, since the movement is greater and the L.T. is advanced by about 20%.

The following conclusions may be tentatively drawn for this plant, with regard to the effect of stimuli above the summer P.T. applied in the winter.

(i) An increase of about 50% over the P.T. for summer is needed to cause the same number of fronds to move.

(ii) The angle of curvature increases with the duration of the stimulus probably in direct proportion within limits.*

(iii) The L.T. falls with the increase in stimulus, but the rate of decrease is not maintained, and it is probable that no increase in stimulus would reduce the L.T. to less than about 20° above that of the summer.

IV—The Diurnal Rhythm of *Asplenium bulbiferum*

The discovery of an annual periodicity in the geotropism of this fern suggested that it might also be subject to a diurnal fluctuation. Most of the experiments recorded in this and other papers of the series were made approximately between the hours of 10.0 a.m. and 7.0 p.m., *i.e.*, stimulation was begun at about the former hour, and response took place usually between 3.0 and 7.0 p.m. Several series were started at 2.0 or 3.0 in the afternoon but the results did not differ from those of experiments begun in the morning. On the night of October 16–17, 1924, two series were begun at 9.0 p.m. and 2.0 a.m. respectively. The first responded normally, but the second showed a marked diminution in sensitivity. It was decided to test this indication quantitatively by carrying out a number of experiments started about 1.0 a.m. Altogether seven series were conducted in four different months, at three different seasons extending over three years. The stimulus was given between the hours of 11.30 p.m. and 3.30 a.m., the conditions of temperature and humidity were carefully kept similar to those of the day time, and the plants used were under special observation during the previous day.

* Although 6° is the *average* angle due to the fact that highly reactive fronds are occasionally met with, 5° is the most frequent angle. On this basis the higher angles recorded for increased stimulus approximate more nearly to direct proportion with the latter.

Although the usual individual variation occurred, in every series a considerable decrease in perceptive power was apparent, and in all but two a slowing down of the mechanism of movement. As it is believed that this is the first time gravitational irritability has been tested at night, the data will be given a little more fully. Tables VIII and IX show the details of experiments made on July 8–9, 1925, and January 8, 1926, which are fairly typical for summer and winter nights respectively. In some cases it was found possible to use identical fronds for day and night

TABLE VIII—JULY 8–9, 11.30 P.M.–2.30 A.M.

Stage of frond	Length cm	Growth increment/day cm	Angle of curvature °	L.T. in hours
L.I.	7·0	2·3	15	6·0
A5–6	10·2	2·1	10	10·5
A2	6·5	0·8	5	5·5
A1	8·6	1·8	5	6·5
L.I.	5·5	1·0	5	7·5
A1–2	6·5	1·2	5	10·1
A1–2	5·9	1·0	4	4·0
A2	5·4	1·2	3	5·5
A6	13·15	1·5	3	6·0
A5	7·7	1·4	3	6·0
A4	9·4	2·2	—	—
A2	6·0	2·0	—	—
A7	12·8	1·4	—	—
L.I.	3·5	1·2	—	—
A4	12·2	1·0	—	—
A5–6	7·5	1·0	—	—
A6	11·6	0·8	—	—
A1–2	6·7	0·6	—	—
A6–7	12·3	0·4	—	—

experiments with only two or three days' interval, so that an even closer comparison could be made. The results of some of these latter are set forth in Tables X and XI. The hours placed after the dates mark the period of time during which or part of which the stimulus was given.

The last two tables are sufficiently striking and scarcely need comment; it may however be emphasized that in Table X while 6 fronds were unresponsive at night and yet moved by day, there is no single case to the contrary; and in Table XI where 6 fronds moved on both days but not at night, there is again no opposite response.

TABLE IX—JANUARY 8, 12.45–2.30 A.M.

Stage of frond	Length cm	Growth increment day cm	Angle of curvature °	L.T. in hours
A1	9·9	1·35	18	6·0
A4–5	14·5	2·0	10	11·0
A1	11·0	1·6	7	5·0
A1	5·5	0·45	5	6·0
A1–2	7·9	1·4	5	6·0
A4	9·6	1·45	5	8·0
A5	12·2	1·35	5	8·0
A1	7·8	0·8	3	6·0
A5–6	8·8	1·1	3	7·0
A5–6	15·4	2·0	—	—
A4	10·1	1·6	—	—
A4	8·7	1·25	—	—
A1	6·5	0·95	—	—
A1	8·9	0·85	—	—
A4–5	10·0	0·8	—	—
A1	7·9	0·8	—	—
A4	6·9	0·8	—	—
L.I.	4·9	0·7	—	—
A1	8·2	0·65	—	—
A1–2	6·8	0·65	—	—
A5	9·7	0·25	—	—

TABLE X—IDENTICAL FRONDS

July 10 2.0–3.30 p.m. Day		July 13–14 11.30 p.m.–1.30 a.m. Night	
Angle of curvature °	L.T. hours	Angle of curvature °	L.T. hours
15	3·5	5	6·5
15	4·5	—	—
15	4·5	—	—
15	5·0	5	5·5
15	5·0	5	5·0
10	3·5	3	6·0
10	4·5	15	6·0
5	3·5	3	4·0
5	5·5	5	10·0
5	5·5	—	—
5	7·0	—	—
5	12·0	—	—
3	5·5	10	10·5
3	7·0	—	—
	7·5	4	11

Note.—Three other fronds did not move on either date.

TABLE XI—IDENTICAL FRONDS

January 4 11.0 a.m.–1.0 p.m. Day		January 6–7 12.45–2.30 a.m. Night		January 8 11.0 a.m.–12.50 p.m. Day	
Angle of curvature	L.T. (hours)	Angle of curvature	L.T. (hours)	Angle of curvature	L.T. (hours)
°		°		°	
5	6	—	—	5	5·5
5	7	—	—	20	5·0
5	7	—	—	5	8·5
3	3	—	—	3	9·0
3	10	—	—	5	10·0
3	12	—	—	3	9·0
5	6	5	6	—	—
5	8	5	6	15	3·5
3	6	5	6	5	5·0
3	10	5	10	—	—
—	—	5	8	6	7·0
—	—	3	6	5	9·5
—	—	—	—	3	8·0

Note.—One other frond did not move on any of the three dates.

Table XII is a summary of the 115 experiments carried out at night, and Table XIII gives a comparative survey of the figures for day and night in summer and winter.

TABLE XII

Date	No. of experiments	Average growth increment/ day cm	Percentage response	Average angle of curvature °	Average percentage advance on "standard" L.T.
1924					
October	18	0·85	56	5·1	27·3
November	19	0·7	47	5·3	40·8
1925					
January	13	1·0	31	6·7	50·0
July	43	1·2	54	7·0	17·5
1926					
January	22	1·1	46	6·6	34·0

V—CONCLUDING REMARKS

Periodicity in plant life is still, as the older observers pointed out years ago (Jost, 1907; Pfeffer, 1903), one of the unsolved problems of biology, and though many facts have been discovered since then, it can scarcely

be said that true knowledge of the subject has advanced. It is not proposed here to enter into a general discussion, but a few remarks on the present work may be allowed. This furnishes an addition from the somewhat neglected field of irritability to the known cases of annual and diurnal rhythm. Table XIII, which summarizes the whole investigation, shows that graviperception falls by 33% at night in both summer and winter, while the decrease in winter over the summer value is only 20% in both day and night. On the other hand the delay in response is 10% (average) at night, but rises to 25% in winter both day and night. From this it seems evident that the mechanism of perception is more affected by the time of day than the season of the year, while the opposite is true for the mechanism of response. Ultimately no doubt perception and response will be seen to be links in a single chain of reactions. Neverthe-

TABLE XIII

Time	No. of experiments	Average growth increment day cm	Percentage response	Average angle of curvature °	Average percentage advance on "standard" L.T.
Day					
Summer	173	1·1	80	6·0	1·4
Winter	287	0·9	64	6·5	32·3
Night					
Summer	43	1·2	54	7·0	17·5
Winter	54	0·9	43	6·1	39·1

less they are convenient terms to express the end members of this chain, and all work in their behaviour may help in the final elucidation of their nature.

Turning to the question of correlation, the rhythm under discussion does not agree exactly with any other known case, though it may be doubted if any periodicity exists which is totally unconnected with others that affect the organism. This is especially true with regard to the great external rhythm of day and night under which all typical plant life has evolved. In nature this means a regular diurnal decrease in light intensity accompanied usually by a similar though not identical alteration in temperature, humidity, and possibly some other factors. Under the experimental conditions of this work, temperature and humidity were carefully controlled and hence could not account for the diurnal behaviour of the fronds. In night work, these factors were usually constant for 24 hours, or more, previous to, as well as during, experimentation; and although a

somewhat lower average temperature in winter months might conceivably accentuate annual rhythm it is quite insufficient to originate it. Neither can alteration in growth rate be held directly responsible for the observed periodicities. A number of records has shown that the growth of these fern fronds is equal day and night (*cf.* Table XIII); and while it is true that the average growth rate is always higher in responding than non-responding fronds, we have abundant evidence to show that graviperception is by no means directly proportional to growth rate. The winter reduction in the latter (*see* Table XIII) is possibly accounted for at least in part by the lower winter temperature, but it seems certain that the lowered irritability is not so. Although it was unfortunately impossible to maintain constant temperature throughout the year, examination of the available data shows that irritability falls in winter independently of growth rate. Looking over winter experiments 40 cases were found in each of which the growth rate was between 1·0 and 1·2 cm per day. These particular fronds gave only 62% response with 34% advance in the L.T.; that is typical winter figures with a summer growth rate. Again, according to Waight (1923), in adolescent stages both perception and response rise and fall while growth is constant (*see* graph, p. 132). A still better proof is afforded by some work of Brain (1933), who finds that in certain seedlings of *Lupinus spp.* there is a winter decline in, or even complete loss of, gravitational sensitivity, though the growth rate remains constant. These facts make it impossible to attribute the winter decline in sensitivity of our plant simply to the lower growth rate, though it is possible that the latter is a factor in the retardation of response. We are then left with the alternation of light and darkness as the outstanding external agents by which the discovered rhythm could be induced, though probably the influence is largely, if not entirely indirect. If it were a direct effect the curve for annual periodicity should follow that for light. But the latter is a symmetrical curve falling in autumn at the same rate as it rises in spring; while, as we have seen, the curves for perception and quickness of response rise slowly in spring and summer (February–August) while falling rapidly in autumn (October). And in diurnal rhythm, we should guard against assuming a direct relation with day and night, since in the well-known sleep movements doubt has recently been cast on their relation to light, the most suggestive and generally accepted cause (de Virville and Obaton, 1922). Many examples are now known of periodicity in plant processes, and we cannot assume that any one of these is independent of any of the others or of external factors; we should rather echo Baranetsky's dictum (1872) " unabhängige Periodicität existirt nicht ". In this connexion I would make one suggestion, namely that

having regard to the accumulating evidence of specificity in physiological processes (Brain, 1933; Dastur, 1925; Prankerd, 1929), it would be advantageous if different periodicities were studied quantitatively *in the same species*. The results might at least be stepping-stones in the elucidation of one of the most characteristic though at present perplexing manifestations of life.

Summary

There is both an annual and diurnal fall in the geotropic reaction time of the fronds of *Asplenium bulbiferum*. Perceptive power as measured by presentation time decreases by 33% at night and 20% in winter, while delay in response (L.T., p. 128) is 10% at night and 25% in winter over summer value.

Increased stimulus, *i.e.*, periods of horizontality above the presentation time, in the winter causes a rise in the angle of curvature and a fall in the latent time; but will not reduce the latter to less than 20% above its summer value.

These rhythms have not been correlated directly with growth or any other internal or external factor.

It is suggested that a knowledge of different rhythms occurring in the same species might throw some light on the problem of the meaning of rhythm.

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The Pigmentary Effector System
VII—The Chromatic Function in Elasmobranch Fishes

By Lancelot Hogben

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Introduction.

It is now firmly established that the coordination of chromatic response in Amphibia is predominantly, if not exclusively, due to the liberation of hormones by reflexes involving visual and skin receptors, and in Reptiles to direct innervation of the pigmentary effector organs (Hogben and Mirvish, 1928; Zoond and Eyre, 1934). Among aquatic vertebrates examples of both types of coordination occur. The bulk of available evidence points to the conclusion that the chromatophores of Teleostean fishes are directly innervated and that the comparatively rapid responses which are exhibited by several species are brought about by simple reflex action. That this is not true of cyclostomes has recently been shown by J. Z. Young (1935) whose experiments demonstrate the archaic phylogenetic character of the control exercised by the Amphibian pituitary gland. From an evolutionary standpoint it would not be surprising to find among physiological mechanisms in Teleostean fishes examples of specialization comparable to the strikingly aberrant features which their anatomical organization displays. On the other hand it would be remarkable if the cartilaginous fishes proved an exception to a rule which applies both to Cyclostomes and to Amphibia. Recent work on the colour changes of Elasmobranchs supports the conclusion that the coordination of colour change in Teleostean fishes is highly specialized. Lundstrom and Bard (1932) have shown that total removal of the pituitary gland in *Mustelis canis* results in a state of pallor which ensues within a few hours after operation, reaching its limit about the twelfth post-operative hour. In their experiments the animals usually succumbed after three or four days with loss of righting reactions. Only a few survived as long as a week. The effect was not produced by removal of the anterior lobe alone, nor by severe traumatization of the hypothalamus. Complete darkening of the pale operated animals followed injections or extracts of ox pituitary and of the pituitary of the fish itself, the

quantity present in the fish gland being greatly in excess of the amount requisite to induce full expansion of the dermal melanophores.

The present investigation, undertaken to throw further light on the evolution of the chromatic function in Vertebrates, is based on several species of Elasmobranch fishes, namely the skates, *Raia Brachiura*, *R. clavata*, *R. maculata*, *R. microcelatus*, the speckled dogfish *Scyllium canicula*, the banded dogfish or nursehound *S. catulus* (*Scylliorhinus stellaris*), and the monkfish *Rhina squatina*. The writer is indebted to Mr. G. A. Steven for invaluable assistance in identifying the various species used. In all these species the pigmentary effector system of the integument, like that of the American dogfish *Mustelis canis*, closely resembles that of Amphibia, and consists of three types of chromatophores which are more or less evenly distributed. These are the epidermal melanophores, larger more richly branched dermal melanophores, and xanthophores containing an orange yellow pigment. The same agencies, in the fishes to be described, evoked or maintained pigment diffusion (" expansion ") of all three types, and the concentration of pigment in the centre of the cell (" contraction ") in all three types. That is to say, the xanthophores of a skate or dogfish which was maximally pale were always fully contracted like the melanophores of both kinds, and the xanthophores of a dark animal were fully expanded. This is true of some—but not all—Amphibia. In general appearance the chromatophores of the species studied are more like those of a Urodele than those of a Teleost.

Normal Reactions

In previous papers the present writer has emphasized the importance of studying the time reactions of chromatic response to normal stimuli before drawing far-reaching conclusions concerning the type of co-ordination involved.

Of the species investigated, *Raia brachiura*, *Raia maculata*, and *Rhina squatina* belong to the same category as *Xenopus*. They show the characteristic " background " response, *i.e.*, complete contraction or expansion of the chromatophores can be produced by keeping them in situations in which the field of vision below the surface of the water is occupied by a light scattering or light absorbing surface. *Raia clavata* on the other hand is like the black axolotl. In normal circumstances the melanophores are always expanded, and the state of pallor induced by operative procedures mentioned below, cannot be evoked by keeping them in a tank with white sides for three weeks. The two species of dogfish (*S. canicula* and *catulus*) studied call for separate comment.

Their chromatophores exhibit a wide range of response analogous to those of *R. brachiura*, but the macroscopic differences are relatively slight. From the account given by Lundstrom and Bard it is not clear whether *Mustelis canis* is a species which exhibits striking responses—microscopic or macroscopic—to its surroundings.

In the confined situations, which such investigations as this necessitate, cartilaginous fishes do not live very long, and it was not possible to secure tank accommodation which would ensure their survival and at the same time provide for the use of standardized and constant illumination as in experiments elsewhere recorded. Nevertheless such observations as were made on the time relations of the normal responses suffice to show that complete equilibrium (*i.e.*, maximal pallor on a white background or

TABLE I—NORMAL REACTIONS OF *Raia brachiura* (10° C)
The Number of Animals on which each Mean Figure for the Melanophore Index is Based is Indicated in Parenthesis

Background	Time of exposure hours	Dermal		Epidermal	
		Normal	Eyeless	Normal	Eyeless
Black	48	4·4 (5)	3·2 (5)	5·0 (5)	3·8 (5)
White	12	2·8 (5)	3·7 (3)	3·6 (5)	4·3 (3)
,,	24	1·8 (5)	3·7 (3)	2·6 (5)	4·3 (3)
,,	36	1·4 (5)	3·7 (3)	2·0 (5)	4·3 (3)
,,	48	1·2 (5)	3·7 (3)	1·6 (5)	4·3 (3)
Black	12	2·8 (5)	3·7 (3)	2·8 (5)	4·3 (3)
,,	24	3·0 (5)	3·0 (5)	3·6 (5)	3·7 (3)
,,	36	4·0 (4)	3·7 (3)	3·7 (4)	3·3 (3)
,,	48	4·3 (4)	3·0 (3)	4·8 (4)	3·7 (3)

maximal expansion of the chromatophores when the animals were kept in tanks with black sides) takes from one to three days at least. In this respect the characteristics of colour change in Elasmobranchs resemble those of colour change in Amphibia and are totally different from those of colour change in the minnow or the chameleon which may give maximal responses in about three minutes.

The progress of the response can be recorded by using the same arbitrary scale of numerical ratings for the configurations of the melanophores used in previous researches (Hogben and Gordon, 1930; Hogben and Slome, 1931) on Amphibia. That is to say a melanophore index of 1·0 represents maximal "contraction" and 5·0 maximal "expansion". The configuration of the pigmentary effector system of the integument is very easy to observe in Elasmobranch fishes by examining the tip of the pectoral or caudal fins through the low power of an ordinary microscope. Table I

records the melanophore indices of a series of young skates (about 15 cm broad between the extremities of the pectoral fins) belonging to the species *R. brachiura* after exposure to white and black backgrounds. Unfortunately for no ascertained reason the removal of the eyes generally proved to be fatal after a week, and only three survived the operation more than three weeks for the purposes of comparison. The behaviour of those that died earlier confirmed the conclusion derived from comparison of the survivors with the responses of the normal animal. That is to say, the melanophores in the eyeless skate, as in blinded individuals of the species *Xenopus laevis*, are neither fully expanded like those of the seeing animal kept on a black background nor fully contracted like those of the seeing animal kept on a white background. In other words the black background response is not merely the absence of the white background response. As in Amphibia the black background and white background responses are both types of chromatic behaviour distinct from that of the animal in which no visual receptors contribute to the response observed

A second similarity which is brought out in this table is that there is a small range of direct response of the melanophores to light independent of the organs of vision. In *Xenopus* the dermal melanophore index of an eyeless animal in bright light is $3 \cdot 2 \pm 0 \cdot 2$, and that of an eyeless animal in dim light, or of a normal animal in darkness, is $2 \cdot 6 \pm 0 \cdot 2$. The greater intensity of light reflected on the skin from the sides of a vessel with white sides, as compared with one with black sides, produces the same type of effect which is indicated by the figures in Table I.

Removal of the Elasmobranch Pituitary Gland

The observation recorded by Lundstrom and Bard (1932) were based on operated animals which did not survive long enough for the wound to heal, and the orifice through which the gland was removed was left open. In the experiments recorded below, the writer attempted to ensure survival by exercising greater care in protecting the wound. The procedure adopted was as follows.

The fish was anaesthetized with urethane dissolved in sea water and then laid with its ventral surface uppermost on a dissecting board with two hooks to dilate the mouth. A triangular flap of mucous membrane over the roof of the mouth was deflected, exposing a pair of prominent transverse blood vessels which lie almost exactly over the middle of the pituitary gland. With this landmark to guide subsequent operations two incisions were made in the floor of the chondrocranium so that a triangular flap of cartilage could be bent backwards (A), in front of, or

forwards (B) behind the transverse blood vessels, fig. 1, to expose the anterior or posterior half of the hypophysis. The whole gland can be removed by a suction tube as prescribed for the Amphibian operation (Hogben, 1923) without further delay or disturbance to the brain. It is better to use route A for the removal of the whole gland with strong suction. The elastic triangle of cartilage if pressed tightly readily slips back in its original place. The cut edges of the flap of mucous membrane are then sewn together with silkworm gut. The whole operation takes less than two minutes, excluding the final stage of sewing the mucous membrane.

The glandular part of the Elasmobranch pituitary has three distinct portions: (*a*) the anterior lobe which extends forwards to the optic chiasma, (*b*) the neuro-intermediate lobe, (*c*) the ventral lobe, which

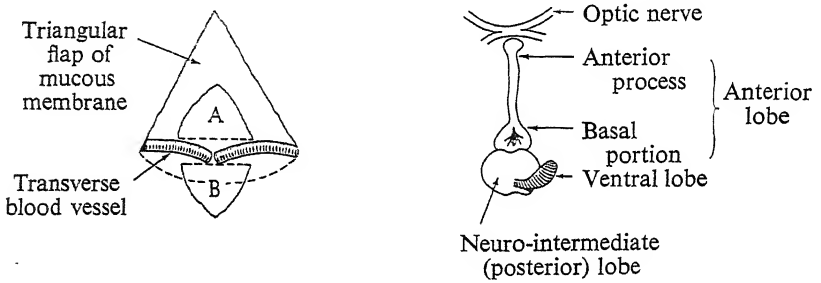

FIG. 1—Operative procedure for removal of the pituitary in the skate.

projects downwards from the last named. In skates the anterior lobe has a broad base histologically equivalent to the pars anterior of higher vertebrates, and a narrow tapering extremity suggestive of the Urodele *pars tuberalis*, and the pars ventralis is free. In dogfishes the anterior lobe is not conspicuously differentiated into a basal and terminal portion, and the pars ventralis appears to be imbedded in the cartilaginous floor of the chondrocranium. For removing the anterior lobe alone the method adopted was to detach it through route A by applying light suction, then snipping through the constriction separating it from the posterior half of the gland, and finally applying strong suction to the anterior end. After a little practice this is as easy as removing the whole gland. To remove the pars ventralis with or without the neuro-intermediate lobe, the procedure is similar, the approach being made through route B, fig. 1.

The first experiments in total hypophysectomy were made on *Scyllium* (both species) and *Rhina*. The former exhibited the characteristic melanophore contraction noted by Lundstrom and Bard (1932). As they proved less convenient for operative purposes than skates, no daily observations

were made. Seven full grown specimens of *Rhina squatina* were subjected to removal of the whole gland. As with all animals so treated, they had been kept in a tank with black sides so that the skin was dark owing to maximal expansion of the chromatophores at the time of operation.

Maximal pallor due to contraction of all three kinds of chromatophores ensued within 24 hours of operation. Two died on the seventh day. three on the eighth. The other two survived for a fortnight when they were killed for autopsy on the internal organs.

Later experiments were all made on skates (four species) with constant microscopic examination of the chromatophores, as recorded in Table II. These animals lived as long as normal controls subjected to the same amount of handling. One animal, which, owing to its large size, was only taken from the tank at intervals of several days, survived for eight weeks in a state of maximal pallor, being then killed for autopsy. Another survived till the thirty-ninth day after operation in the same condition.

None of these animals showed the slightest interference with their normal swimming movements. Apart from the striking chromatic effect which is seen in figs. 2 and 3, they appeared to be normal.

Removal of the Separate Lobes of the Hypophysis

It is not feasible to remove the posterior, *i.e.*, neuro-intermediate lobe without removing the pars ventralis at the same time. The interpretation of the effect produced by the former operation therefore depends on that produced by the latter alone. Table III records the dermal melanophore indices of eight skates from each of which the pars ventralis alone was removed. The first four (*R. clavata*) were originally dark as this species normally is. Three survived more than 10 days with no detectable effect. The other four (*R. brachiura*) were kept on a white background after operation and should (Table I) have assumed pallor within three days if they were not pale at the time. The record shows that they did not develop such extreme pallor as the normal animal, and their reaction would seem to be more sluggish (*vide* especially No. 23).

This apparently sluggish reaction calls for comment for a special reason. In all the skates from which the whole pituitary gland was removed the skin exhibited a distinctly pink flush due presumably to peripheral vaso-dilation. This accompaniment of hypophysectomy is always noticeable in Amphibia, being originally discovered by Krogh (1922) and frequently observed by the writer. As Krogh has pointed out, it provides clear evidence for the view that the pressor component of

TABLE II—TOTAL HYPOPHYSECTOMY—DERMAL MELANOPHORE INDICES—BLACK BACKGROUND

Species	No.	Days after operation																		
		0	1	2	3	4	5	6	7	8	9	10	12	14	17	18	21	30	39	40
Raia brachiura	300	4·0	—	1·5	1·5	1·0	1·0	1·0	1·0	1·0	D									
,, ...	301	4·0	—	1·0	1·0	1·0	1·0	1·0	D											
,, ...	303	4·0	—	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	—	—	1·0	—	—	1·0	D	
,, ...	306	5·0	—	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	D							
,, ...	307	4·0	1·0	—	1·0	—	—	—	1·0	—	1·0	—	1·0	1·0						
,, ...	308	4·0	1·0	—	1·0	—	—	—	1·0	—	1·0	—	1·0	1·0						
,, ...	309	5·0	1·0	—	1·0	—	—	—	1·0	—	1·0	D								
,, ...	310	5·0	—	—	—	—	—	—	—	—	—	1·0	—	—	—	—	1·0	1·0	—	1·0
,, ...	311	5·0	—	—	1·0	—	—	—	—	—	1·0	—	—	—	1·0					
Raia maculata	305	4·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0								
,, ...	5	5·0	—	—	—	—	—	2·0	—	—	1·0	—	—	D						
Raia microcelata ..	77	5·0	—	1·0	—	1·0	—	1·0	—	—	—	1·0	1·0	D						
,, ..	525	5·0	1·0	—	—	—	—	1·0	—	—	—	—	—	1·0						
Raia clavata	90	5·0	—	—	1·0				1·0	—	1·0	1·0								
,, ...	91	5·0	2·0	—	1·0				1·0	—	1·0	—	D							

TABLE III—REMOVAL OF THE VENTRAL LOBE—DERMAL MELANOPHORE INDICES

Species	Back-ground	No.	Days after operation																	
			0	1	2	3	4	5	6	7	8	9	10	11	13	14	17	21	26	28
Raia clavata	Black	51	5·0	—	4·0	—	4·0	—	—	—	—	—	—	5·0	—	D				
,, ...	,,	56	5·0	—	5·0	—	3·0	—	—	—	—	—	—	4·0	4·0	—	D			
,, ...	,,	78	5·0	—	5·0	—	5·0	D												
,, ...	,,	89	5·0	—	5·0	—	5·0	—	—	—	—	—	—	D						
Raia brachiura	White	4	2·5	—	2·0	2·5	—	—	2·5	2·5	2·5	3·0	2·5							
,, ...	,,	10	3·0	—	2·0	2·0	—	2·0	2·5	2·0	3·0	3·0	3·0							
,, ...	,,	12	4·0	—	2·0	2·5	—	2·5	2·0	2·0	2·0	2·0	2·0	—	—	—	2·0			
,, ...	,,	23	3·0	—	—	—	—	—	—	3·0	—	—	—	—	—	—	—	2·0	2·0	2·0

posterior lobe extracts in the higher vertebrates has an active physiological role in the intact animal. Although the pressor activity of extracts prepared from the Teleostean hypophysis has been demonstrated by Herring (1913) and by Hogben and de Beer (1925), no evidence of a pressor component could be found in that of the Elasmobranch. Since the oxytocic substance is present in both, Herring interpreted his observations to signify the separate identity of the oxytocic and pressor substances, a conclusion

FIG. 2—Two adult *Rhina squatina*. On left normal, on right operated animal 10 days after removal of whole pituitary gland. Both kept continuously in black tank.

amply confirmed by subsequent evidence derived from purely chemical technique though, for reasons pointed out by Hogben and de Beer, insufficiently justified by his original data. The present writer was formerly inclined to regard a purely negative result with suspicion, which has been strengthened by the fact that all the skates from which the pars ventralis alone was removed showed the pink flush referred to above. It was especially noticeable at the edge of the pectoral fin of the dark animals.

If, then, a pressor substance is present in the pars ventralis, as this fact suggests, previous failure to detect it in extracts of the Elasmobranch

pituitary is easily explained. In skates the ventral lobe adheres to the membranous floor of the brain case and that of dogfishes is imbedded in the latter, so that it is invariably left behind, unless special precaution is taken to remove it along with the rest of the gland. Should this surmise be supported by preparation of special extracts from the pars ventralis. the sluggish behaviour of the chromatophores in skates from which only this lobe had been removed recalls the sluggish reaction of Amphibian

FIG. 3—Two adult *Raia clavata.* On left 10 days after removal of whole pituitary gland. In right 10 days after removal of the pars ventralis.

colour response when peripheral vaso-motor disturbance has been produced by transection of the C.N.S.

When the neuro-intermediate lobe is removed together with the pars ventralis (Table IV) extreme pallor results. Eight operations on *R. brachiura* kept in a tank with black sides are recorded. Four of the animals which survived over 10 days were killed for autopsy. One lived over three weeks. The impression gained was that the contraction of the melanophores was even more extreme than as seen when the same species

is kept for long periods on a white background, or when the whole gland is removed. Judged by the arbitrary scale of melanophore indices adopted, the pallor was at least as great as that ensuing after total hypophysectomy.

Removal of the anterior lobe alone had no noticeable effect on animals which were kept in conditions to ensure maximal expansion of the chromatophores. If performed on pale animals kept in conditions to ensure pallor on normal specimens, the result is analogous to the effect of removing the anterior lobe (pars anterior and pars tuberalis) of *Xenopus*. That is to say (Table V) the animals become dark and in several cases were more conspicuously dark than normal animals kept on a black background. Out of 11 cases melanophore expansion was maximal in five, and remained so till death or autopsy in one case 26 days after operation. One animal only showed partial expansion.* The drop in the melanophore indices of Nos. 2 and 3 on the twenty-second day was recorded when the animals were moribund owing to failure in the aeration of the tank in which they were kept. The significance of these data will be discussed later.

Injection of Extracts and Implantation of the Gland

It has already been shown (Hogben and Winton, 1922) that the melanophore stimulant or "B" substance which evokes expansion of Amphibian melanophores is present in the pituitary gland of fishes as well as in the neuro-intermediate lobe of land vertebrates. A series of implants of (*a*) the neuro-intermediate lobe, (*b*) the anterior lobe of dogfishes and skates, was made with *Xenopus* as test material. All implants of the neuro-intermediate lobe produced melanophore expansion in pale animals. Implants of anterior lobe produced negative or relatively slight results in pale animals. A slight drop in the melanophore index resulted from anterior lobe implants when dark animals were used, but it was not so pronounced as to justify any definite conclusion.

In view of the effect produced by removing the neuro-intermediate lobe in Elasmobranchs, these experiments suggest that one and the same hormone ("B" substance) produced by the neuro-intermediate lobe in Elasmobranchs as in Amphibia is responsible for the "black background response" and for the maintenance of melanophore expansion in species which normally remain dark, like *Raia clavata* or *Amblystoma tigrinum*. It has been shown by Hogben and Gordon (1930) that the "B" substance

* Histological examination of the gland shows that anterior lobe tissue does not end abruptly where the constriction separates the two lobes morphologically.

TABLE IV—REMOVAL OF NEURO-INTERMEDIATE (POSTERIOR LOBE)—DERMAL MELANOPHORE INDICES—BLACK BACKGROUND

Species	No.	Days after operation													
		0	1	2	3	4	5	6	7	8	9	10	16	17	24
Raia maculata	14	5·0	—	2·0	—	—	—		—	—	D				
,, ...	160	4·0	—	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	—	2·0	D
Raia brachiura	10	4·0	—	—	1·0										
,, ...	12	5·0	—	—	1·0	—	—	—	—	—	—	1·0			
,, ...	13	5·0	—	—	1·0		—		D						
,, ...	15	5·0	—	—	2·0	—	—	—	D						
,, ...	18	5·0	—	1·0	—	—	—	—	—	—	D				
,, ...	19	5·0	—	2·0	—	—	—	—	—	—	1·0	—	1·0		
,, ...	20	5·0		2·0	—	—	—	—	—		1·0	—	1·0		
,, ...	21	5·0	—	2·0	—	—	—		—		1·0		1·0		

TABLE V—REMOVAL OF ANTERIOR LOBE—DERMAL MELANOPHORE INDICES—WHITE BACKGROUND

Species	No.	Days after operation																			
		0	1	2	3	4	5	6	7	8	9	10	12	14	15	17	20	21	22	24	26
Scyllium canicula	26	2·0	—	—	4·0	—	—	—	4·0	—	—	D									
,,	27	2·0	—	—	4·0	—	—	—	4·0	—	—	5·0	—	—	5·0						
Scyllium catulus	28	2·0	—	—	4·5	—	—	—	4·5	—	—	D									
Raia brachiura ..	1	3·0	—	—	—	—	—	—	—	4·0	—	—	D								
,, ..	2	3·0	—	—	—	—	—	—	—	4·5	—	—	—	4·0	—	—	—	—	3·0 }	dying	
,, ..	3	2·5	—	—	—	—	—	—	—	4·5	—	—	—	4·0	—	—	—	—	3·0 }		
,, ..	6	2·0	—	—	5·0	—	—	—	—	—	5·0	—	D								
,, ..	7	2·0	—	—	5·0	—	—	—	—	—	5·0	—	—	—	—	5·0					
,, ..	245	2·0	—	—	—	—	—	4·5	—	—	—	—	5·0	—	—	—	5·0	D			
,, ..	260	3·0	—	—	—	—	—	5·0	—	—	—	—	5·0	—	—	—	5·0	—	—	—	5·0
,, ..	266	2·0	—	—	—	—	—	3·0	—	—	—	—	3·5	—	—	—	3·5	—	D		

which acts on Amphibian melanophores is identical with neither the pressor nor with the oxytocic component of posterior lobe extracts. Lundstrom and Bard (1932) were able to induce elasmobranch melanophores to expand after injection with vaso-pressin but not after injection with oxytocin. They therefore concluded that the Elasmobranch hormone is identical with the pressor substance. This inference is not permissible, because the Amphibian " B " substance also separates out with the pressor component in the method used to prepare commercial extracts with pressor or only oxytocic action. As shown by Dale and Dudley (1929) pressor and oxytocic activity are both destroyed by exposure to the action of cold normal sodium hydroxide. To decide whether the substance which evokes expansion of Elasmobranch melanophores is a separate entity, an extract was, therefore, prepared by mixing a 10% ox pituitary gland extract with one and a half times its own volume of 2N sodium hydroxide. The mixture was neutralized after two hours, and evoked maximal expansion of the melanophores of small skates (15 cm from one pectoral extremity to the other) in dilutions equivalent to 0·02 cc of the original 10% extract of ox gland. The " B " substance responsible for the Elasmobranch black background reaction is, therefore, different from the pressor and oxytocic principles, and there can be little doubt that the mechanism of the black background response is essentially the same in Elasmobranch fishes and in Amphibia.

Discussion

The experimental data may be briefly summarized as follows.

(i) In Elasmobranch fishes as in Amphibia which exhibit a " background " response the condition of the melanophores in the eyeless animal is intermediate between maximal expansion, resulting from prolonged exposure to a black, and maximal contraction resulting from exposure to a white background. These responses take about three days to reach completion. There is also, as in Amphibia, a narrow range of " primary " or direct response of the melanophores, which tend to expand more in bright light.

(ii) Removal of the whole pituitary gland or of the neuro-intermediate lobe alone completely abolishes the black background response and produces extreme pallor even in species which normally remain dark on a white background. As with Amphibia the black background response can be reproduced in normal pale or hypophysectomized Elasmobranchs by injection of posterior lobe extracts free of oxytocic or pressor activity.

(iii) Removal of the pars ventralis alone abolishes neither the white nor the black background response. Removal of the anterior lobe alone abolishes the white background response at least temporarily and apparently permanently.

The following conclusions may be drawn with confidence from these data.

(i) The black background response and the white background response depend on separate receptive elements, or separate coordinating systems, or upon a combination of both. From the observed time relations it is intrinsically improbable that peripheral innervation of the melanophores plays any significant role in these changes. In other words they are either due to stimulation and inhibition of the secretory activity of one and the same endocrine organ, or to the liberation of two distinct hormones with antagonistic effects.

(ii) The black background response of Elasmobranchs like that of Amphibia, as also the maintenance of the dark condition by species which do not respond to a white background, is due to a hormone produced by the neuro-intermediate lobe, which is reflexly stimulated by light acting on the retina. This hormone is distinct from the pressor and oxytocic substances produced by the same organ.

In the light of evidence which will be published shortly it is clear that the black background response in Amphibia is brought about by the action of light on the ventral region or *floor* of the retina, whereas the white background response depends on the stimulation of photoreceptors in the dorsal and peripheral part of it. If this is also true of Elasmobranch fishes it would seem that we must choose between two hypotheses: (*a*) that the stimulation of ventral photoreceptors reflexly *activates* and stimulation of dorsal photoreceptors reflexly *inhibits* the production of the B substance by the neuro-intermediate lobe; (*b*) that the stimulation of ventral photoreceptors reflexly activates the production of B (which evokes melanophore expansion) and that the stimulation of dorsal photoreceptors reflexly stimulates the production of a second hormone " W ", which evokes contraction of melanophores. Previous work (Hogben and Slome, 1931) and later experiments, which will shortly be published, have led the writer to adopt the second hypothesis to account for the phenomena of Amphibian colour change. According to the evidence obtained in enquiries based on Amphibia " W " is associated directly or indirectly with the pars tuberalis which in *Xenopus* as in Elasmobranchs is not morphologically distinct from the pars anterior. The results of removing the anterior lobe of Elasmobranch fishes support the same hypothesis though they do not conclusively establish its truth. Unfortunately the

difficulties of such enquiries on marine animals have prevented the completion of other experiments which, it is hoped, will throw further light on the issue, when opportunity for continuing the work is available.

It is a great pleasure to acknowledge the courtesy and consideration with which Dr. E. J. Allen, F.R.S., placed the necessary facilities for this investigation at the writer's disposal. Acknowledgment is also made to Mr. C. Wingfield and Mr. S. Alexander for assistance in the care of the animals.

Summary

The pigmentary effector system of the integument in Elasmobranchs consists of dermal xanthophores, dermal melanophores, and epidermal melanophores.

Two species of *Scyllium* (*catulus* and *canicula*), *Rhina squatina* and two species of *Raia* (*maculata* and *brachiura*) exhibit contraction of all three types when kept in a container with white sides, and expansion when kept in one with black sides. The " background " response, which is macroscopically striking in *Rhina squatina* and *Raia brachiura*, but not so in the two species of *Scyllium*, is visually controlled.

The background response develops gradually and requires several days to reach its maximum. Some species of Elasmobranchs, *e.g.*, *Raia clavata*, like the black axolotl, do not exhibit a pronounced white background response.

Within 24 hours after operation, total removal of the pituitary gland in all the species mentioned results in complete pallor, which persists till death. The operation can be carried out without any interference with swimming movements or other evident signs of disturbance or damage to the C.N.S., and the fish will survive at least two months.

The same result follows removal of the neuro-intermediate lobe with or without the pars ventralis.

Removal of the pars ventralis alone does not abolish the white or black background response.

Posterior lobe extracts free of pressor and oxytocic activity induce complete expansion of Elasmobranch pigment cells.

Removal of the anterior lobe alone appears to abolish the white background response. The indefinite demarcation of this lobe may be the explanation of partial recovery which ensued in one case.

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The Pigmentary Effector System

VIII—The Dual Receptive Mechanism of the Amphibian Background Response

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Introduction

In a previous contribution (Hogben and Slome, 1931) evidence was brought forward to show that the white background response does not depend on the same mechanism of coordination as the black background response, which is produced by reflex liberation of a hormone ("B" substance) of the pars intermedia in the pituitary gland; and experiments pointing to the existence of another internal secretion ("W" substance), connected directly or indirectly with the activity of the pars tuberalis, were described. The existence of separate receptor components of the retina controlling the two systems was left for subsequent enquiry. Of two possible hypotheses concerning the nature of the receptive mechanism, the most likely one is illustrated diagrammatically in fig. 1. In normal

situations, when an animal is illuminated on a black background, light can only fall on the floor of the retina. If it is aquatic, the maximum divergence of any two rays which strike the eye is twice the *critical* angle for air and water, so that in the absence of reflexion of rays from surrounding objects below the surface of separation all rays will presumably be brought to a sharp focus in shallow water. There are thus three distinct possibilities which arise from the way in which the animal is illuminated if, as in *Xenopus*, the eyes are situated on the top of the head: (*a*) in darkness no part of the retina is stimulated, the same being true of the eyeless animal; (*b*) when the animal is exposed to a black background only a sharply localized region of the retina is stimulated: (*c*) when the animal is exposed to a white background the whole of the

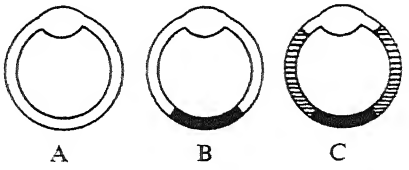


FIG. 1—Diagram to illustrate illumination on black background (B), white background (C), and the same eye in darkness (A). The eye is drawn facing upwards towards the source, as in aquatic animals with dorsally placed eyes.

retina is illuminated owing to the scattering of rays in all directions from the surroundings. For convenience of description the usual black background situation will be described hereafter as one in which only the "floor" of the retina is stimulated, and the white background situation as one in which the *floor* and the "*periphery*" of the retina are both stimulated together. If then, the receptor elements of the floor and periphery initiate different systems of reflex arcs the phenomena of the background response in Amphibia and Reptiles may be interpreted as follows. In Reptiles we may suppose that stimulation of *floor* reflexly excites the melanophores to expand, while stimulation of *peripheral* photoreceptors excites them to contract, being presumably prepotent in the final common path. In Amphibia two alternatives may be considered: (*a*) that floor elements reflexly excite liberation of "B" and that peripheral photoreceptors, being prepotent, reflexly inhibit liberation of "B"; (*b*) that floor elements reflexly excite liberation of "B", and peripheral photoreceptors reflexly excite production of the antagonistic substance "W" in quantity sufficient to over-ride the effect of "B".

The crucial test of the truth of the general hypothesis that the floor and

peripheral elements of the retina initiate different processes of coordination was suggested by Keeble and Gamble (1904–6) in their experiments on Crustacea. If it is true, a normal animal illuminated from *below* in a black tank with a white top should react in exactly the same way as a normal animal when illuminated in a black tank from above. On the other hand, a normal animal illuminated from below in a black tank with a black top should react like an *eyeless* animal in the same situation, because the floor elements would not be subject to stimulation. The

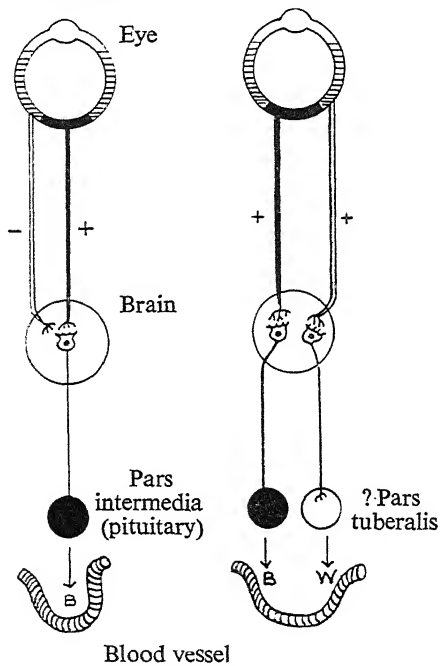


FIG. 2—Diagram to illustrate the one hormone and two hormone hypotheses of chromatic coordination in Amphibia (or Elasmobranch fishes).

experiment may be varied as indicated below. In order to obtain significant results two classes of precautions must be carefully observed. One is that the physical dimensions of the tank must not exceed certain limits, since the maximal divergence of two rays is rigidly fixed when an animal is illuminated from above. The other is that there must be no air-water interface to permit reflexion of the incident rays downwards. Aside from the fact that no bubbles must be allowed to collect, this condition presents a practical difficulty if the animal has to come to the surface to breathe.

A fact which gives the hypothesis under consideration *prima facie* plausibility is that normal black background responses in aquatic animals

are most pronounced when the water is very shallow. On the view which we are about to explore, the optimum conditions for a black background response must be defined by a limiting divergence of the rays incident on the eye. Above a certain limiting depth depending normally on the amount of surface exposed to illumination from above, the maximum divergence of any two rays is fixed by the critical angle. Above this level it makes no difference how shallow the water is. In carrying out the *experimentum crucis* outlined above Keeble and Gamble (1904–6) obtained negative results but do not say whether the precautions specified were taken. In a previous enquiry by Hogben and Slome (1931) analogous experiments on *Xenopus* were carried out with inconclusive but generally negative results, and the experiments were discontinued owing to the writers' departure from South Africa. At the suggestion of Sir Henry Dale it was decided to explore this possibility with greater care.

TABLE I

Animal	Background	Illumination	
		Intense	Dim
Normal	Black	$4 \cdot 8 \pm 0 \cdot 2$	$4 \cdot 2 \pm 0 \cdot 2$
Eyeless	Black	$3 \cdot 3 \pm 0 \cdot 3$	$2 \cdot 7 \pm 0 \cdot 3$
Normal	White	$1 \cdot 6 \pm 0 \cdot 2$	$1 \cdot 3 \pm 0 \cdot 2$
Eyeless	White	$3 \cdot 3 \pm 0 \cdot 3$	$2 \cdot 7 \pm 0 \cdot 3$

For the clarification of what ensues, Table I briefly summarizes the range of primary and secondary responses of the dermal melanophores in *Xenopus laevis*. The mean figures refer to the arbitrary ratings used in previous researches (Hogben and Gordon, 1930; Hogben and Slome, 1931) to describe the configuration of the pigment cells ranging from $1 \cdot 0$ for complete "contraction" to $5 \cdot 0$ for maximal dispersion. The primary or direct response is seen in the differences recorded along each horizontal row and the secondary or visual response appears in the differences recorded along each vertical column. The melanophore index of the seeing animal kept in complete darkness is the same as that of an eyeless animal in dim light, *i.e.*, $2 \cdot 7 \pm 0 \cdot 3$.

BACKGROUND RESPONSE WITH INFERIOR ILLUMINATION

The type of tank used for testing the effect of illumination from below is seen in fig. 3. It was $29 \cdot 5$ cm square, and $38 \cdot 5$ cm high. The floor was of plate glass. Below it was an obliquely placed white screen

illuminated by a 25-watt lamp placed about 2 feet away. Two tanks of similar dimensions were made one with sides painted a dull black, the other with sides painted white. The submerged roof just under the level of the water was reversible, being white on one side and black on the other. Six toads were used in every experiment. The depth of the animals could be varied by a movable glass plate. In each experiment the melanophore index was recorded at 24-hour intervals.

In one series of experiments the arrangement to permit aerial respiration was a narrow alley of metal painted black, placed as in the diagram. The recorded melanophore indices of the animals after two days' exposure in a tank with black sides with the treatment specified is shown in Table II. The temperature was 14° C. All figures represent the average of six toads.

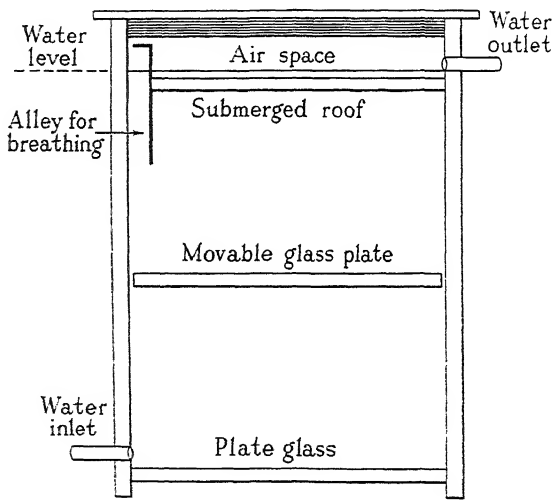


FIG. 3.

TABLE II—ILLUMINATION FROM BELOW IN TANK WITH BLACK SIDES

Top	Distance cm	Melanophore index
White	11	4·75
Black	11	3·8
White	11	4·7
White	14	4·7
Black	14	3·7
White	14	4·7
White	18	4·7
Black	18	4·1
White	18	4·6

In this series of experiments there was a consistent drop of the melanophore index when the black top was substituted for a white one, and this was also true of toads kept in a white tank, *e.g.*, at 14 cm 1·7 to 1·5, at 18 cm 1·7 to 1·3, and at 22 cm 1·7 to 1·4, a result which was repeated successfully. That this shift was not due to any diminished intensity of direct illumination on the skin, *i.e.*, to the intervention of the primary response, was tested repeatedly by placing eyeless animals in the same tanks with black and white tops, *e.g.*, in the black tank at 22 cm the figures for eyeless animals were, successively,

White top	2·8
Black top	3·0
White top	3·0

As far as they go this series of experiments encouraged the belief that the hypothesis under consideration may be correct. The results are not decisive, because in no case did the substitution of a black top for a white one in the tank with black sides lower the melanophore index to the level of the eyeless animal. The fact that the shift was least at the greatest depth provided the clue to previous failure. At the greater depth it seemed inevitable that there would be more escape of reflected light from the interface at which the toads were permitted to fill their lungs. In another series of experiments this defect was rectified by substituting a breathing hole of about 1·5 cm diameter covered loosely with black cloth, firmly fixed at its edges. This hung down in the water preventing direct contact of air and water except when a toad thrust its nostrils into the orifice, and the slackness of the cloth just allowed access to the air. The results then obtained, when the toads were free to settle at the bottom of the tank, are given below, Table III, each mean figure based on six animals after three days' exposure.

TABLE III—ILLUMINATION FROM BELOW IN TANK WITH BLACK SIDES

White tops	Black tops
4·5 ± 0·25	2·8 ± 0·3
4·7 ± 0·2	2·5 ± 0·2
3·85 ± 0·3	2·8 ± 0·2
4·8 ± 0·1	3·1 ± 0·3

In this series the result of cutting off direct illumination of the floor of the retina is to reproduce exactly the condition of the eyeless animal. The experiments recorded were carried out in 1931. Since then they

have been repeated from time to time. All the results are summarized in Table IV in which it is assumed that illumination from below in a tank with a white *top* signifies stimulation of the *floor* of the retina, and in a tank with white *sides* stimulation of the periphery of the retina. The positive sign signifies excitation. The intensity of light throughout was sufficient to evoke the primary responses.

TABLE IV

Floor of retina	Periphery	Melanophore index
+	−	4·5
+	+	1·8
−	−	3·0
−	+	1·3

BACKGROUND RESPONSES IN MONOCHROMATIC LIGHT

The foregoing data point to the existence of two categories of photoreceptors, one of which is morphologically localized in the floor of the eye, being so situated that it is only brought into play when rays converge upon it from directly overhead. It seemed not unlikely that the " floor " receptors which initiate the black background response and the " peripheral " receptors which initiate the white background response might be sensitive to different ranges of wave-length. To test this possibility the background responses of toads exposed to monochromatic light were recorded.

The toads were exposed in black and white containers placed in closed chambers fitted with Wratten filter windows. The catalogue numbers, range, maximum wave-length intensity, and percentage transmission of the filters used, are given in Table V.

TABLE V

	Range 99%	Maximum intensity	Transmission %
Blue (49)	400–490	460	0·5
Green (62)	520–570	530	4·0
Yellow (73)	560–620	570	3·3
Red (29)	610 to invisible	700	6·6
" Infra-red (88) " ..	700 to invisible	—	—

To allow for differences in transmission, different intensities were tested by varying the distance of the source from the window filters. In Table VI the intensity ratio of dim and bright light was 3 : 50. The melanophore indices were recorded after four days' exposure at 14° C.

TABLE VI

Colour	White background		Black background	
	Dim light	Bright light	Dim light	Bright light
Blue	1·0	1·0	3·3	3·0
Green	1·0	1·0	3·0	3·3
Red	1·3	1·0	5·0	4·3
Infra-red	2·0	2·7	3·0	2·7

These observations point to the conclusion that the photoreceptors which initiate the black and white background responses are sensitive to different wave-length ranges. The "floor" receptors are specially sensitive in the red end of the spectrum.

FURTHER ANALYSIS OF TIME RELATIONS

Two alternatives, fig. 2, demand reconsideration in the light of the new information set forth above. These are: (*a*) that stimulation of floor elements reflexly *excites* liberation of "B" and stimulation of peripheral elements reflexly (and prepotently) *inhibits* liberation of "B": (*b*) that stimulation of floor elements reflexly excites liberation of "B" and stimulation of peripheral elements reflexly excites the liberation of a second hormone "W", in quantity sufficient to over-ride "B".

With these two alternatives before us the time relations of the background responses acquire a new significance. If hypothesis (*a*) is correct the absolute amount of "B" in the circulation is necessarily higher when the melanophore index is higher, and lower when the melanophore index is lower. If hypothesis (*b*) is correct a low melanophore index may be due to increased amount of "W" relative to "B" compatibly with a greater absolute quantity of "B" in the circulation, and a high melanophore index may be due to a diminished quantity of "W" relative to "B" compatibly with a smaller absolute amount of "B" in the circulation. If hypothesis (*a*) is correct *transition from one state to another must therefore involve liberating more or excreting more of the one hormone (B), and must necessarily take longer than a transition which involves building or excreting less.* If hypothesis (*b*) is correct transition from one state to another may involve a single process of liberation (or excretion) or two competing processes, and in general we may plausibly postulate that equilibrium will be reached more slowly when two opposite reactions compete simultaneously.

The issue may be put more forcibly if we use round figures for the melanophore index, eliminating the effect of the primary response. For

instance, transference of an animal kept on a white background in light to a black background in light involves a shift from 1·0 to 4·0 when equilibrium is reached. On the other hand transference of an animal kept on a white background in light to complete darkness only involves a shift of 1·0 to 2·5, and if the one hormone hypothesis is correct less " B " is discharged in the process of reaching equilibrium than would be discharged if the animal had been transferred to a black background in light. All the relevant responses may be set forth thus:—

(*a*) From white to black background (1·0 → 4·0).
(*b*) From white background to total darkness (1·0 → 2·5).
(*c*) From black to white background (4·0 → 1·0).
(*d*) From black background to total darkness (4·0 → 2·5).
(*e*) From darkness to white background (2·5 → 1·0).

If we now use the symbols, t_a, t_b, etc., for the times occupied in reaching equilibrium under the conditions specified as (*a*), (*b*), etc., the one hormone hypothesis implies three consequences which can be tested very simply. These are:

(i) $t_a > t_b$.
(ii) $t_c > t_d$.
(iii) $t_c > t_e$.

With reference to these the data submitted in a previous communication were not wholly conclusive. To obtain unequivocal evidence three additional precautions were taken in a new set of experiments: (*a*) the responses were observed at a lower temperature (14° C), which slows the reactions and thus exaggerates significant differences; (*b*) the slight diurnal rhythm seen in darkness (Hogben and Slome, 1931) was eliminated by taking all day to day records in darkness at the same hour (noon); (*c*) the rate at which equilibrium was attained by normal animals in darkness was compared with the rate at which equilibrium was attained by normal animals kept on white or black backgrounds when the eyes were removed.

The relevant information is given in fig. 4. All the animals had previously been kept on a white or black background for three to four weeks. Eyeless toads which have been kept in the laboratory two or three years after operation have a melanophore index of 2·7 ± 0·3 in dim light. If normal specimens previously kept in bright light on a black background (m.i. = 4·6) are transferred to the dark room at 14° C they reach this level about 12 hours later, and oscillate about it thereafter. Previously dark toads (m.i. = 4·7 in the figure) after removal of the eyes

reach the same level by the fourth or fifth day after operation. Toads previously kept on a white background (m.i. = 1·3–1·4) if transferred to the dark room or subjected to ophthalmectomy at 14° C and kept in dim light do not reach the equilibrium level after 21 days. On the other hand pale toads (m.i. = 1·3) transferred to a black background in dim light reach the equilibrium level (4.1) about 12 hours later, surpass it, and settle down to a steady state within five days. It seems therefore that at this temperature (14° C) a shift in the melanophore index from 1·3 to 2·7 takes at least 40 times as long as a shift from 1·3 to 4·1, a result which is inexplicable if only one hormone is involved in the background responses.

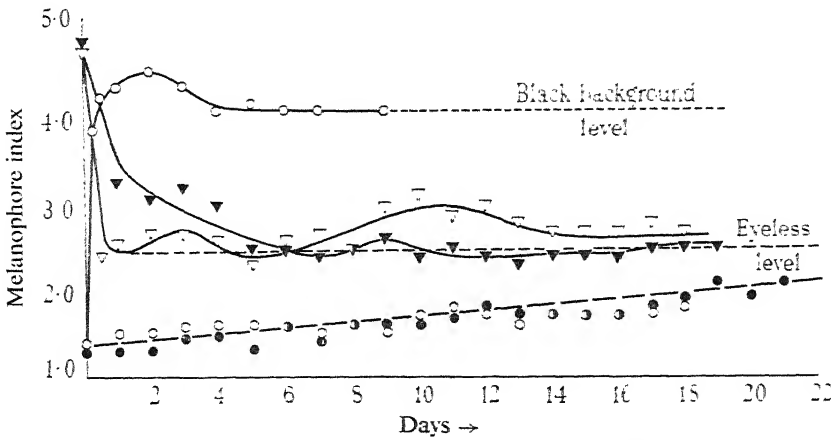


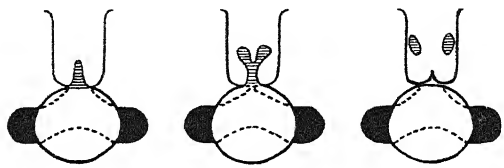
FIG. 4—Graph showing the time relations of colour change in *Xenopus*.

REMOVAL OF SEPARATE LOBES OF THE PITUITARY

The evidence advanced in a previous communication (Hogben and Slome, 1931), to support the dual nature of the coordinating mechanism involved in Amphibian colour change, rested on three results of operative procedure, depending on differences in the relations of the lobes of the hypothesis in different species of Anura. In *Rana* and most other Anura, the pars tuberalis is (fig. 5) represented by two small plaques which lie fixed to the tuber cinereum some distance away from the anterior lobe, so that removal of the pars anterior alone or of the pars anterior and posterior lobe together does not involve removal of the pars tuberalis. In the adult *Xenopus*, the pars tuberalis retains the embryonic condition, *i.e.*, it is represented by a single forward lip continuous with the pars anterior, so that removal of the pars anterior alone or of the pars anterior

and posterior lobe together involves removal of the pars tuberalis. The evidence for the existence of a " W " substance produced by the pars tuberalis (or by some organ whose activity depends on the pars tuberalis) in contradistinction to the " B " substance which is produced by the pars intermedia was:—

(*a*) Removal of the anterior lobe (without removal of the pars tuberalis) in *Rana* did not affect colour change. Removal of the anterior lobe along with the pars tuberalis in *Xenopus* completely abolished the white background response, *i.e.*, the toads remained maximally dark.



A, Adult *Xenopus* B, *Bufo* at the time of net amorphosis C, Adult *Rana*

(Pars tuberalis: shaded; pars intermedia: black)

FIG. 5.

(*b*) After removal of the pars anterior and pars posterior without removal of the pars tuberalis, *Rana* was *less* sensitive to " B " extracts than the normal pale frog. After removal of the pars anterior and pars posterior, together with the pars tuberalis, *Xenopus* was *more* sensitive to " B " extracts than the normal pale toad.

(*c*) The pallor of *Rana* after removal of the pars anterior and pars posterior without removal of the pars tuberalis was more intense than that of the normal animal in equilibrium with a white background. The removal of the entire gland (including the pars tuberalis) in *Xenopus* did not produce a pallor as intense as the pallor of the white background response.

The argument based on a comparison of two different species which offer different opportunities for removal of the gland would be reinforced if analogous operations could be carried out on one and the same animal. As regards (*a*), previous experiment had already shown that removal of the pars anterior in *Xenopus* does not abolish the white background response, unless the forward lip (pars tuberalis) is also detached. In

subsequent attempts to produce pallor by implantation of, or injection of extracts made from the pars tuberalis consistent success has not been attained. In itself this is not a decisive objection to the existence of the hypothetical "W" substance for several reasons. Owing to the portal circulation of the mammalian pituitary gland it is difficult to prepare from its separate lobes extracts which contain only the hormones produced by them, while the pars tuberalis of *Xenopus* itself is exceedingly minute compared with the pars intermedia. There is actual evidence that extracts prepared from any part of the gland will contain some of the very stable and highly active "B" substance, and there is presumptive evidence that no extract is likely to contain a large quantity of the hypothetical "W" substance which may well prove to be less stable. These considerations are greatly strengthened by two others. Since the white background response subsides into the black background response rapidly, the hypothetical "W" substance is presumed to be destroyed or eliminated more rapidly than "B"; and since the black background response passes into the white background response so slowly we are led to suppose that the latter is built up gradually rather than discharged from an accumulated excess already present.

However the absence of definite evidence of this kind makes it imperative to explore every alternative avenue, and we have therefore undertaken two new classes of experiments:—

(*a*) Comparison of the pallor resulting from the removal of the posterior lobe alone with pallor resulting from removal of the whole gland including the pars tuberalis.

(*b*) Comparison of the sensitivity of the same two classes of toads to "B"-containing extracts.

The removal of the posterior lobe alone is more easy to carry out in *Xenopus* than in *Rana* by a modification of the original method given by Hogben (1923), since the anterior lobe remains attached by the pars tuberalis to the tuber cinereum, when it is lifted up to permit the removal of the lobe which lies beneath. In earlier experiments we have found that the mean m.i. of a very large number of animals from which the whole gland has been removed settles down to a value of about 1·8 irrespective of background in fairly bright illumination. In the same conditions the mean figure for normal animals on a white background may be taken to be 1·4. After removal of the posterior lobe only, a dozen animals kept under observation for six weeks uniformly exhibited maximal (pin point) "contraction" of the dermal melanophores, having

a mean m.i. = 1·0, irrespective of background. When the sensitivity of the three classes to "B"-containing extracts was compared the results confirmed earlier work, in showing that the normal animal kept on a white background was more tolerant than the toad made pale by complete removal of the gland, and elicited the additional information that the tolerance of toads made pale by the removal of the posterior lobe alone was still greater. A protocol is given in the legend below fig. 6. The results of both experiments fulfil exactly the requirements of the hypothesis advanced. A normal pale animal is supposed to contain "W" in sufficient quantity to over-ride the "B" in the circulation. If the pars

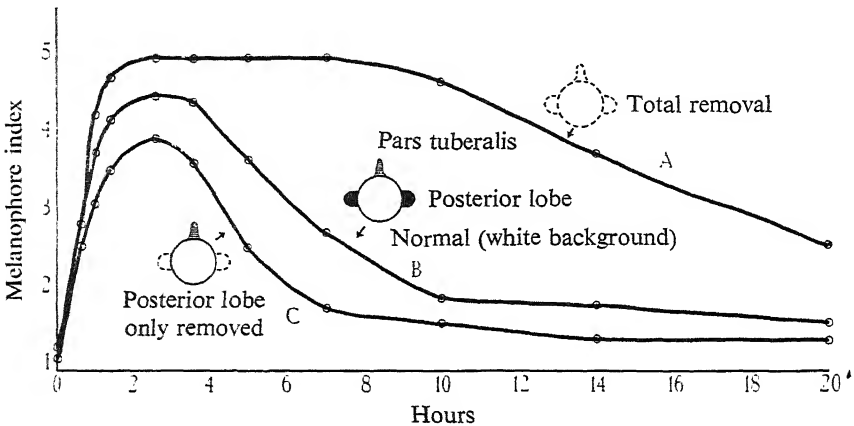


FIG. 6—Tolerance of 3 series of 6 toads to pituitary extracts. The animals were selected for the same degree of pallor and of weight, being 30 ± 2 gm. Each received 0·075 cc of a 10% extract of ox posterior lobe deprived of all pressor and oxytocic activity by the method of Dale and Dudley (1929). Temperature 14° C.

tuberalis is the seat of the "W" substance, total removal of the whole gland reduces the absolute quantity of both "B" and "W" to zero. An animal from which only the posterior lobe has been removed should have some "W" but no "B" in the circulation.

[*Note added in proof, March* 20, 1936—This differential tolerance, which is utterly unintelligible, if the one-hormone hypothesis is correct, fits in with the fact that removal of the pars tuberalis abolishes the white background response. Thus the "B" substance injected into a pale toad deprived of the intermediate lobe alone has to counteract a full complement of "W" with no antagonistic "B" in the circulation. The "B" substance injected into a normal pale toad has to antagonize a certain excess of "W" over the normal complement of "B". The "B" substance

injected into an animal from which both lobes have been removed has not got to neutralize the effect of any "W" substance at all.]

Hence the pallor and tolerance of the three classes should be as set forth diagrammatically in fig. 7.

As already explained in a previous paper on the chromatic function in

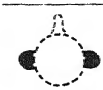
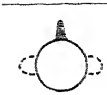
	Melanophore index		Sensitivity to B extracts
	White background	Black background	
Normal	1·4	4·5	— —
A.L. alone removed	1·4	4·5	....
Total removal	1·8	1·8	— — —
 A.L. — tuberalis removed	5·0	5·0	....
 P.L. alone removed	1·0	1·0	—

FIG. 7—Chart summarizing present and previous results on the effect of hypophysectomy on the chromatic function of *Xenopus*.

Xenopus laevis, it should be emphasized that the conclusions here stated are solely concerned with the behaviour of the *dermal melanophores* which are the principal, but not the sole, agency of colour change. In general the epidermal melanophores seem to behave like them: but the epidermal melanophores and especially the xanthophores are often difficult to distinguish in the translucent web. The writers have never been able

to complete a satisfactory account of their role on this account. That they do make a significant contribution to the macroscopic effect is clearly shown by injection experiments. Although a melanophore index of 4·0–5·0 invariably corresponds to maximal darkening of the skin, when it is recorded as a response to normal stimuli (" background "), as also to extracts prepared from the whole pituitary gland, a melanophore index of 5·0 evoked as in the last experiment by injection of pressor, oxytocic-free posterior lobe extracts prepared by the method of Dale and Dudley (1929) is consistent with a very low grade of visible darkening barely in excess of what is ordinarily associated with an m.i. of 3·0. This discrepancy may be interpreted in one of two ways. Either the threshold of the different kinds of pigmentary effector organs to the " B " and " W " substances is different, or the behaviour of the xanthophores is regulated by some additional secretion which has not yet been identified. At present no data decisively dismiss the first and more economical suggestion.

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Summary

Experiments in which the effects of illumination from above and below, and of illumination with monochromatic light of different wave-lengths have been compared, show that the white and black background responses of *Xenopus* depend on distinct localized retinal elements.

The time relations of the chromatic function so far as they depend on the dermal melanophores are inconsistent with the assumption that the two types of photoreceptors respectively activate and inhibit one and the same mechanism of coordination.

Further experiments on removal of the separate lobes of the gland indicate that, while the black background response depends on the " B " substance or " melanophore principle " of the pars intermedia, the white background response is brought about by a second hormone (" W " substance) secreted by the pars tuberalis or by some organ whose functional activity depends on the presence of the latter.

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Studies on the Nature of the Amphibian Organization Centre

III—The Activation of the Evocator

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[PLATE 7]

1—INTRODUCTION

The first attempts to produce a capacity for induction in tissue which is normally incapable of performing such an action were made by Spemann and Geinitz in 1927. They grafted a fragment of presumptive ectoderm into the organization centre of another embryo, and, removing it a few hours later, found that it had been "infected" with the inducing capacity of the tissues by which it had been surrounded. The experiment inevitably suggested that the inducing capacity is the property of a chemical substance which had diffused out of the organizer tissue into the grafted ectoderm fragment. A similar hypothesis could be used to explain the observation of Mangold and Spemann (1927) that in normal development the presumptive neural plate acquires inducing capacity

at the same time and in proportion as it is underlain and determined by the mesodermal organizer. The first suggestion that the non-inducing parts of a Urodele gastrula themselves possess an organizing capacity, which is masked but only awaits activation or release, emerged in the work of Dürken (1926), Bautzmann (1929, *a*, *b*), Kusche (1929), and Holtfreter (1931), and attention was first drawn to it by Huxley (1930). The German authors showed that if fragments of the gastrula are " interplanted " into the body cavity or optic vesicle of older larvae, they may develop into something other than their presumptive fate, and in particular, presumptive epidermis or neural plate may develop into various mesodermal derivatives such as notochord or muscle. Huxley pointed out the similarity between this phenomenon, which was called *bedeutungsfremde Selbstdifferenzierung*, and the results of isolating parts of the axial gradient system of lower organisms, which have been particularly described by Child (summaries 1928, 1929). An isolated part of an axial gradient system reconstructs a " dominant region "; and Huxley suggested that we could account for *bedeutungsfremde Selbstdifferenzierung* by supposing that an isolated part of a gastrula reconstructs the dominant region, *i.e.*, the organization centre.

In the spring of 1932 one of us (C. H. W.), while on a visit to the laboratory of Dr. O. Mangold in Berlin for the purpose of learning the technique of amphibian operations, attempted to carry the matter a step further. If Huxley's explanation were correct, one would have to suppose that a capacity for behaving like a " dominant region ", that is, for inducing, is latent in the presumptive ectoderm, and this capacity should become manifest when the ectoderm changes into a dominant region after isolation. The following experiment was therefore made to test this point. Fragments of presumptive ectoderm from a young gastrula were interplanted into the eye-cavity of Anuran tadpoles, from which the eye-ball had previously been removed. After two days the interplanted tissue was removed and grafted by the *Einsteck* method into the blastocoele of young newt gastrulae, to discover whether they were capable of inducing the formation of neural plate. Three sets of controls were made. In one set organizing tissues were interplanted for two days and then tested to see whether their inducing capacity had been impaired, in the second set organizing tissue was isolated for two days in Holtfreter solution, and then tested, and in the third set presumptive ectoderm was isolated for two days in Holtfreter solution and tested for inducing capacity.

The tests of the interplanted ectoderm were negative, no good inductions being obtained. Too much weight should not be laid on this finding since the operative technique employed was not very good, but a plausible

explanation of this failure, if it is a real phenomenon, will emerge in a moment. The ectoderm isolated in Holtfreter solution also failed to induce, as might have been expected. The organizing tissue retained its inducing capacity after isolation either in the eye-cavity or in salt solution; this fact, which was also found by Holtfreter (1933, *a*) in a much larger material, disposes of Altekrüger's (1932) suggestion that inducing capacity is dependent on the structural integrity of the organizer tissues.

Since 1932 several lines of work have conspired to throw doubt on Huxley's explanation of *bedeutungsfremde Selbstdifferenzierung*. Holtfreter (1931) showed that the phenomenon did not occur in tissues isolated in salt solution, which immediately raised the possibility that its occurrence in tissues isolated in the body cavities of larvae may be due to the influence of the body fluids. This explanation has been made enormously more probable by the discovery that the active agent in neural induction is a chemical substance which is very widely distributed and occurs in most adult animals. This discovery removes the original ground for supposing that the ectoderm cells contain an organizing principle in a masked condition, and allows us to account for the *bedeutungsfremde Selbstdifferenzierung* as due to the diffusion of active substances into the tissue. The failure of induction by the tissues isolated in the eye-cavity, mentioned above, is probably to be explained by the hypothesis that a sojourn of two days is not long enough for the penetration of an adequate amount of the active substance into the tissues; although in normal development the determination of the neural plate takes a shorter time than this, yet isolated tissues can retain their reactivity for as long a period as two days (Waddington, 1935), and it may well be that induction in the eye-cavity is a slow and lengthy process.

But at the same time as the original grounds for assuming the presence of a masked inductive capacity in the ectoderm were destroyed, new and more convincing grounds for the same hypothesis were discovered. Holtfreter (1933, *b*) showed that the non-inducing parts of the amphibian gastrula become inducing after any treatment which leads to the death of the cells. It can thus be taken as certain that the non-inducing tissue contains the active principle of the organization centre, the evocator (Needham, Waddington, and Needham, 1934), in some form in which it cannot exert its normal influence.

With the resuscitation of this hypothesis, Huxley's original suggestion of the parallelism between the organization centre and the "dominant region" of an axial gradient becomes once more worth testing. The axial gradient cannot, of course, be directly the active agent in performing an induction, since that active agent has been shown to be one or more

chemical substances, but it is possible that the substance, which is present throughout the whole embryo, is liberated or activated in one particular region, the organization centre, by reason of a gradient system.

The metabolic nature of an axial gradient is a matter which is still under discussion, but its protagonists (*see* Watanabe and Child, 1933) are wont to suggest that it is a gradient of " metabolic rate ", in particular of respiratory rate. It might therefore be argued that anything which would raise the respiratory rate of isolated pieces of gastrula ectoderm would thereby liberate or activate the masked evocator which they contain. The first part of the material to be reported here is based on this line of argument. An attempt was made to raise the respiratory rate of isolated fragments of ectoderm by the use of dyes which act as respiratory catalysts, and the treated ectoderm was then tested for inducing capacity. An investigation was also made to determine whether a gradient of respiratory rate is characteristic of the gastrula in normal development, and in the second part of the paper data are given on the respiratory rate of the dorsal lip region and of the ventral ectoderm.

In the spring of 1934 work was begun with the respiratory catalyst (methylene blue) experiments. Later in the same year there appeared a paper by Brachet (1934, *b*) who had been led, quite independently, to formulate a similar hypothesis, and who had therefore measured the oxygen consumption and the carbon dioxide output of the organization centre as compared with that of the ventral ectoderm. Carbon dioxide production was determined directly on fragments of dorsal lip and ectoderm isolated in a small closed space containing bicarbonate solution and cresol red; the acid production being followed colorimetrically. The oxygen consumption, however, was measured indirectly by taking the oxygen uptake of eggs from which either the dorsal lip or an equivalent amount of ventral ectoderm had been removed. It was thought advisable, therefore, to continue work on oxygen consumption, measuring it directly by micro-manometric means. Early in 1935 a sufficiently sensitive micro-respirometer was constructed (J. N. and J. B.) and later on during the laying season measurements were accordingly carried out on fragments of dorsal lip and ventral ectoderm (C. H. W.). It is hoped to make further progress along these lines in future seasons.

2—The Effect of Respiratory Catalysts on Gastrula Ectoderm

Fragments of ectoderm, including both presumptive epidermis and presumptive neural plate, were removed from the young newt gastrula

and explanted into Holtfreter solution containing the dyestuffs. The dyes used were methylene blue, cresyl blue (Barron and Hoffman, 1930), and 4-6-dinitro-*o*-cresol (Dodds and Greville, 1933).* The explants were left for two days in the solution before being implanted into the blastocoele of young newt gastrulae. The explants had usually rounded up into compact masses by the time the implantation was carried out, and they were then cut across so that they might enter into more intimate contact with the host tissues. The masses of tissue were not usually deeply stained by the dyestuffs. Only the cresyl blue seemed to penetrate readily into the cells, the tissues explanted into solutions of this substance becoming homogeneously light pink in colour during the period of cultivation. Any dead cells adhering to the fragment were coloured pale blue, and were removed before the implantation. The tissues in dinitro-*o*-cresol were entirely normal in coloration. Those in methylene blue were for the most part also not visibly coloured, but in some masses of tissue there were diffuse pale patches of bluish coloration, particularly on the original inner surface of the tissue. The effect of the methylene blue could however be seen in another respect. The explants in this substance secreted an abnormally large amount of mucus, becoming quite sticky, so that they adhered firmly to the needles used in the operation. It was also found, in the tissues sectioned at later stages, that an abnormal number of pigment granules had been formed, but this was not visible under the binocular at the time of implantation.

A few of the explants in methylene blue were cultivated in isolation for longer than two days. Many of the masses of tissue appeared to differentiate into epidermis just as they would have done in plain Holtfreter solution; they acquired the typical crinkled surface and began to rotate by ciliary movement. Others, however, remained as solid smooth-surfaced masses, on which no ciliary beat developed. They have not been investigated in sections, since the sections of the implanted fragments can give the same information.

Description of Specimens

CM–1. Ectoderm from a young Axolotl gastrula was kept for two days in Holtfreter solution with M $\times$ 10^{-4} methylene blue, and then the explants, which were stained light blue, were subdivided and the fragments implanted into young Axolotl gastrulae. The embryo was fixed two

* The value of using cresyl blue consists in the fact that it does not form methaemoglobin from haemin compounds in the cell (de Meio, Kissin and Barron, 1934); and 4: 6-dinitro-*o*-cresol has the effect of stimulating glycolysis as well as respiration.

days later in the stage when the neural folds were just closing. A large thickening of the ectoderm was visible above the graft, and the thickening bore a groove which sections show to be a small typical neural plate. The implant tissues are still young but seem to be more neural in appearance than ordinary explanted ectoderm;* fig. 2, Plate 7.

C29*a*–4. Axolotl ectoderm was kept two days in M $\times 10^{-4}$ methylene blue and then implanted into young *Triton taeniatus* gastrulae. It was light blue in colour and very sticky from the secretion of mucus. In the neurula stage, two days after the operation, an induced neural plate appeared, joined to the anterior end of the host plate, and continuing slightly to the right of the main axis on to the belly of the embryo. The main mass of the implant, which was more heavily pigmented than the surrounding tissues, lay under the end of the induced plate furthest removed from the host plate. In the sections of the embryo, which was fixed in the tailbud stage on the third day, the induced tube can be seen leaving the host tube between the eyes and running down on to the belly. The implant tissues are neuralized, particularly in the region nearest the host tube, where a definite neural tube with a lumen is present; fig. 4, Plate 7.

C30*a*–2. The graft consisted of Axolotl ectoderm, kept two days in M $\times 10^{-5}$ methylene blue; it was neither visibly stained nor particularly sticky at the time of implantation into *T. taeniatus* hosts. The embryo was fixed in the long tailbud stage on the fourth day, when a large thickening could be seen. The sections show that this thickening contains a large induced neural tube; fig. 3, Plate 7; while the implant is also neuralized and in places contains a definite tube with a lumen.

D34*b*–4. The graft consisted of *T. alpestris* ectoderm, kept for two days in M $\times 5 \cdot 10^{-5}$ methylene blue. It was slightly blue and rather sticky when implanted into the *T. alpestris* host. Fixed on the third day in the mid tailbud stage. There is a considerable thickening of the host tissues, but no neural tube, or even neuralization. In the implant, however, the tissues which face inwards towards the endoderm are neuralized, and in one place there is a short but very definite piece of neural tube; fig. 5, Plate 7.

* *I.e.*, elongated cells with oval nuclei are seen in random distribution.

TABLE I

	Exp. No.	Conc.	Implant: Neural tube	Implant: Neuralized	Implant: Epidermal	Host reaction: Neural tube	Host reaction: Neuralized thickening	Host reaction: Epidermal thickening or nothing	Totals used: Sectioned	Totals used: Life
Methylene blue	CM–1	M × 10^{-4}	0	? 1 (young)	0	1	0	0	1	1
	C25	,,	0	0	5	0	0	5	5	6
	C29	,,	0	1	3	1	0	3	4	4
	M31	,,	0	0	0	1	0	0	0	1
	C92	,,	0	1	0	1	0	0	1	1
	C102	,,	0	0	4	0	0	4	4	7
	CM2	M × 10^{-5}	0	? 2 (young)	0	1	0	1	2	2
	C26	,,	0	0	1	0	0	1	1	7
	C30	,,	1	2	2	1	1	3	5	7
	C42	,,	0	0	5	0	0	5	5	8
	C56	,,	0	0	2	1 (in life)	0	1	2	8
	C90	,,	0	2	2	0	0	4	4	4
	D34	5M × 10^{-5}	2	0	2	0	0	4	4	4
		Totals	3	9	26	7	1	31	38	60
Dinitro-*o*-cresol	C43	4M × 10^{-6}	0	0	7	0	0	7	7	10
	C50	,,	0	0	4	0	0	4	4	17
		Totals	0	0	11	0	0	11	11	27
Cresyl blue ..	D6	M × 10^{-5}	0	0	13	0	0 [1 balancer]	13	13	13

3—The Oxygen Uptake of the Organization Centre and of Ventral Ectoderm

The measurement of the oxygen uptake of fragments of the amphibian gastrula presents certain difficulties by reason of the smallness of the quantities involved. We have used a modification of the respirometer described by Gerard and Hartline (1934). The measurement is effected by watching, under the microscope, the movement of a drop of oil along a capillary in which the tissue is placed. In our apparatus, fig. 1, three capillaries were used simultaneously, one as a thermo-barometer, the second for the organizer tissue, and the third for the ventral ectoderm. Each capillary was slightly enlarged at one end, the edges of which were

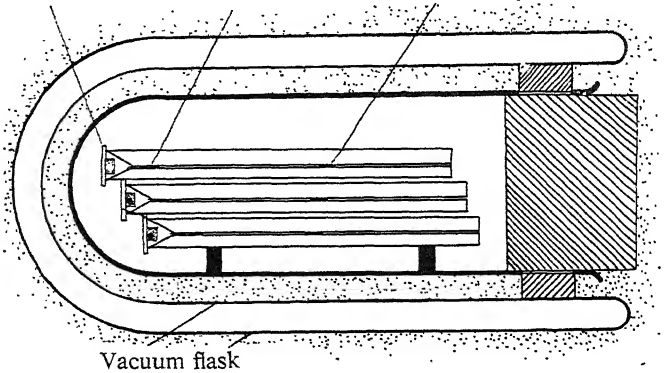

Fig. 1.

ground to a plane, so that it could be closed by a small glass plate sealed on with vaseline. Attached to this glass plate is a small glass cup which projects into the cavity of the swollen end of the capillary; this glass cup is made from glass tubing which is drawn out to a fairly coarse thin walled capillary in an ordinary flame, and then drawn out again and modelled to a flat-bottomed shallow cylinder, working with watch-makers' forceps in a microflame. The cup is sealed on to the glass plate with de Khotinsky cement. The swollen ends of the capillaries were roughly of the same volume, and so were the cups, the volume of the latter being about 1 cu mm.

In preparing for an experiment, the capillaries were first cleaned thoroughly and dried in an air current. A drop of paraffin stained red with Scharlach was then introduced through the non-swollen end and allowed to flow up and down the tube until the walls were thoroughly

wetted; if the drop did not move freely the capillary was cleaned again. A small triangular piece of filter paper, or a thin cotton thread, was then soaked in 2% sodium hydroxide, freed from surplus moisture and inserted into the swollen end of the tube. The tissue fragments were usually dissected out an hour before the experiment began, so that by the time the tubes were ready to receive them they had rounded up to some extent and the wound edges had healed. They were then placed in the cups, which were filled with Holtfreter solution, dried off, and sealed on to the tubes with vaseline. The tube was then held vertically to make certain that the vaseline joint was gas tight and did not allow the drop to move under the influence of gravity. If all was well, the three tubes were placed one above the other in the holder, which is made of two discs of hard rubber, in which a suitable slot is cut, held together by two stiff wires. The vaseline joints were again tested, and the holder and tubes were then slid into a large test-tube into which the rubber discs fit fairly closely. The test-tube was then closed with a rubber bung, which, increasing the barometric pressure inside, caused the oil-drops to move along the capillaries; care was needed to ensure that after these manipulations the three oil-drops were approximately vertically over one another, so that they could all be seen together in one field of the telescope. The test-tube was then fixed into a cylindrical Dewar flask, the space between the test-tube and the walls of the flask being filled with water, which was taken from the large tank in which the whole apparatus was then immersed. In most of our experiments this tank stood in a constant temperature room kept at 25° C. After allowing half an hour for the equalization of temperature the position of the oil-drops was read on a telescope fitted with a micrometer eye piece. As oxygen is consumed, the oil-drop moves towards the swollen end of the tube, the carbon dioxide being absorbed by the alkali. The readings of its position are corrected for temperature and barometric changes by comparison with the readings of the control thermo-barometer tube which contains no tissue.

The first series of experiments, Table II*a*, were made with eggs of *Rana temporaria*. A rectangular piece of tissue was removed from a young gastrula immediately above the dorsal lip, and another piece taken from the same egg from the ventral ectoderm above the position in which the ventral lip of the blastopore would form at a later stage. In these experiments no attempt was made to weigh the fragments of tissue used, but they were compared under the dissecting microscope and cut as carefully as possible to the same superficial area. The pieces differed in size considerably in different experiments. The figures show that a consistent difference in the oxygen consumption was found, the material

TABLE II

a—Rana temporaria, at room temperature

Experiment	Hours	O_2 consumption cu mm/hr		O_2 consumption cu mm/mg/hr	
		Org. centre	Ventral ect.	Org. centre	Ventral ect.
18	16·7	0·045	0·032		
19	6·6	0·081	0·065		
20	17·25	0·0545	0·047		
21	18·8	0·027	0·022		
	5·4	0·026	0·019		
22	4·25	0·030	0·024	0·178	0·184
	8·2	0·034	0·028	0·20	0·215
	Average	0·0425	0·034		

b—Triton alpestris, at 25° C

Experiment	Hours	O_2 uptake cu mm/mg/hr	
		Org. centre	Ventral ect.
26	2·8	0·13*	0·18
	4·8	0·14*	0·18
27	3·25	0·22	0·21
	5·5	0·22	0·22
28	6·0	0·40†	0·38†
29	3·0	0·27	0·25
	16·25	0·20	0·22
30	3·5	0·25	0·26
	6·75	0·23	0·22
31	13·4	0·18	0·16
32	3·9	0·22	
	16·4	0·24	
33	5·5	0·24	0·26
34	15·5		0·17
	Average	0·227	0·212

* Some cells were dead, giving too high a value for the weight, and too low a value for O_2 uptake per mg. Excluded from average.

† Readings taken by an assistant, some error probably made, excluded from average.

from the dorsal lip (organization centre) having a slightly higher consumption than the ventral material.

The method of cutting out equal sized pieces of tissue under the microscope cannot, however, be regarded as satisfactory, since even if the pieces are equal in area, they may differ in thickness, which is more difficult to control. The dry weights* of the tissue fragments were therefore obtained.

Only one weighing was made in the *Rana* series before the experiments had to be given up at the end of the egg-laying season. It showed that in this case, in which the oxygen consumption of the organizer fragment was, in the usual way, higher than that of the ventral ectoderm, the organizer fragment was just sufficiently heavier to account for the difference. It seems probable, therefore, that in the other experiments also the greater oxygen consumption of the organizer fragments is to be explained not by a greater uptake per unit weight but by the difference in weight between pieces of equal area from the dorsal lip region and the ventral ectoderm. Such a difference was in fact suspected before the weighing was made, since one can see that large yolky cells are firmly attached to the dorsal lip fragments, while the ventral fragments were taken from a region in which the ectoderm separates cleanly from the underlying endoderm.

Experiments were then started on the embryos of *Triton alpestris*. All the fragments whose oxygen consumption was measured were weighed, and there was therefore no need to cut out pieces of equal area. Several pieces were used together, the dry weight in each cup being usually between 0·3 and 0·5 mg. The organizer tissue and the ventral ectoderm were always taken from the same embryos, so that there should be no disturbance due to individual differences between the eggs used. The oxygen consumptions, calculated as cu mm/mg/hour, show a moderate amount of variation from one experiment to the other, but in each experiment the two values measured tend to be fairly close together, so that the variation between experiments can probably be ascribed to some general influence such as temperature change. In the experiments in which two readings were taken after different intervals of time, the values have not changed very much, except for the organizer tissue of experiment 29. The two average values for the whole series of experiments do not differ significantly. We therefore find that in *Triton alpestris* there is no significant difference in the oxygen consumption per unit weight between

* The weighings were very kindly performed by Mr. S. Smith, to whom we wish to offer our thanks.

the organizer tissue and the ventral ectoderm, a conclusion which we also saw to be probable in the case of *Rana temporaria.*

4—Theories of the Evocator

Previous work has revealed several different treatments, which when applied to competent gastrula ectoderm cause the tissue to develop into neural tissue. These treatments consist in the exposure of the ectoderm to various substances.

From the investigations leading up to the paper of Waddington, Needham, Nowiński, and Lemberg (1935) the following were found: (A1) unsaponifiable digitonin-precipitable fraction of adult liver; (A2) ethereal extract of crude glycogen prepared from adult mammalian liver To these Waddington and Needham (1935) added (A3) certain synthetic oestrogenic hydrocarbons. The finding of Fischer and Wehmeier (1933) that crude glycogen possessed activity was explained admittedly by (A2) above, but they still (1934) claim as active: (A4) thymonucleic acid; and (A5) muscle adenylic acid. In their most recent paper, Fischer, Wehmeier, Lehmann, Jühling, and Hultzsch (1935) report positive results with (A6) oleic, linolenic, and other higher liquid fatty acids. In their opinion, the induction is brought about by the stimulus of a large number of acid substances, and they speak of a "Säure-reiz".* Unfortunately during the past three years no photographs of their histological findings have been published,† and these will be awaited with interest. Finally, from the work of Barth (1934) we must add (A7) crude kephalin preparations; and from the present work (A8) methylene blue.

If we make the probable assumption that the activity of crude kephalin preparations is due to some impurity of the type mentioned in (A1), (A2), or (A6), we may summarize this list by grouping the active substances into the three classes of sterol-like compounds for 1, 2, and 3, acids for 4, 5, and 6, and methylene blue.

We also know of another set of processes, which, when applied to gastrula ectoderm, cause that tissue to become capable of evocating the formation of neural tissue from a second piece of ectoderm. These processes are: (B1) invagination through the blastopore, which operates in normal development; (B2) treatment with organic solvents; (B3) boiling, drying, and freezing; (B4) being evocated, since neural tissue

* In this concept there seems to be some obscurity, since the buffering power of the tissues would be expected to neutralize acid ions.

† With the exception of two open neural plates by Fischer (1935).

which has been formed by evocation is then capable of itself performing an evocation; (B5) from the present work we must add, treatment with methylene blue.

We see that exposure to methylene blue is the only type of treatment which occurs in both lists, and we can only discuss the significance of methylene blue on the basis of a general theory as to the relations between the treatments in the two lists. Any such theory must at the present time still remain largely speculative, and in selecting, among the many alternatives which might be suggested, certain possibilities to serve as a working hypothesis, our choice will be guided chiefly by the criterion of simplicity.

We may begin by the consideration that the naturally occurring evocator which occurs in the invaginated material at the dorsal lip of the blastopore, must be " specific ". That is to say, it must be some definite compound; we cannot suppose that the evocation is in some eggs performed by one substance, and in other eggs of the same species by another substance. It is clearly simplest to assume, as a working hypothesis, that this " specific " substance falls into one of the groups mentioned in our first list, *i.e.*, is either a sterol-like substance or an acid. We shall discuss later how such substances might be supposed to be liberated in normal development by the process B1.

The change brought about in non-inducing ectoderm by treatment with organic solvents (B2) may also be assumed to be a liberation of acids or sterol-like substances. There is also no theoretical difficulty in assuming that a similar liberation occurs under the influence of the treatments B3. To account for the activation of the evocating powers of ectoderm by the process of being evocated (B4) we have an even simpler possible hypothesis; it is only necessary to suppose that the evocation consists in the diffusion into the ectoderm of one of the substances mentioned in list A, and that the evocating power which the ectoderm then exhibits is due to the subsequent diffusion of the same substance out again.

When we consider the mechanism by which methylene blue both performs an evocation and causes the ectoderm on which it acts to become evocating, we cannot avoid considering two contrasting possibilities. The first is that methylene blue, in bringing about these two results, acts in a way similar to the natural evocator; that is to say, it diffuses into the ectoderm and acts directly as a stimulus to neural differentiation, and then may subsequently diffuse out again into new ectoderm, where it again acts as a stimulus to neural differentiation. The second is that the methylene blue activates, in ectoderm which is exposed to it, one of the substances of list A which was present in some inactive state within the

ectoderm, and that then this substance A can act as a stimulus to neural differentiation both on the tissue in which it has been activated and, by diffusion, on other ectoderm brought in contact with it. There is no immediate way in which we can arrive at a definite decision as to which of these possibilities is actually realized. But the adoption of the second alternative allows us to formulate a much simpler working hypothesis than is possible if the first alternative is adopted. The first alternative supposes that methylene blue acts directly as a stimulus to neural differentiation, just as does the natural evocator. But it would be ridiculous to suppose that methylene blue *is* the natural evocator. Therefore we are committed to the assumption that there is more than one substance which can act directly as a stimulus to neural differentiation. The second alternative, on the other hand, allows us to suppose that the effect of methylene blue is exerted through the intermediacy of either an acid or a sterol-like compound, and therefore does not add to our list of active substances. But it does still more in the way of simplification. It raises the possibility that a compound may cause ectoderm to develop into neural tissue, not by acting directly as a stimulus of neural differentiation, but by activating, within the ectoderm, the naturally occurring stimulus which is already there in a masked form. It therefore suggests that we need not consider even two different classes of active substances, acids and sterol-like compounds, but that it is possible that one of these types of substance acts by unmasking a substance of the other class.

This line of argument allows us to make the very simple hypothesis that there is only one class of related substances which act as direct stimuli to neural differentiation. But it brings us face to face with a fundamental methodological difficulty which it will not be easy to overcome. It may be stated in this way. Competence to form histologically neural tissue and a morphologically recognizable neural tube cannot be separated from the masked evocator. Free evocator can exist without competence, as in adult tissues and the invaginated mesoderm, but the opposite condition has not so far been observed. It is therefore formally impossible to maintain, in any case where a given substance is used as the successful stimulus for an induction, that this substance has any constitutional relation to the substance normally at work in the dorsal lip of the blastopore, for it can always be held that the substance tested simply unmasked or activated the natural evocator already present in the competent tissue.* In so doing, it would be acting in the same, but

* Analogous difficulties may exist in other branches of biology. Thus pharmacologists seek to account for the parasympathetic action of pilocarpine by supposing that it stimulates cells to produce acetylcholine (*see* the review of Ing, 1935).

a much more gentle, way, as boiling the ventral normally non-inducing ectoderm or treating it with organic solvents.

The difficulty of deciding whether the acids unmask the sterols or vice versa appears to be considerable, but we believe the question of *dosage* is here very important. We can probably accept the principle that, other things being equal, the larger the necessary dose, the more damage will be done to the tissue, and the more likely it will be that an unmasking of the natural evocator is taking place; whereas the smaller the necessary dose, the nearer the substance probably stands in chemical constitution to the evocator itself. From this angle we may consider the substances which have been found to possess evocator activity.

In general the dose has been large. Thus Barth used mixtures of 50% kephalin with kaolin, and in the last paper of Fischer and his collaborators we read that nucleoprotein fractions were only active if implanted as such, and not in combination with agar or gelatin jellies. Similarly Fischer and Wehmeier used up to 20% crude glycogen in gelatin or egg-white, and, from their descriptions, the amounts used of thymonucleic acid and of muscle adenylic acid must have been even greater. The higher liquid fatty acids were used finely emulsified in agar jellies at a concentration of 5% of the acid. The experiments with methylene blue must count as having a high dosage, since the dye was available, indeed, to the cells in excess.

Only in the experiments with unsaponifiable ether-soluble fractions have the doses been really small. Thus in experiment C27*b*, referred to in the first paper of this series, the amount of solid matter in the ethereal extract of glycogen was so minimal as to be hardly visible when the ether was evaporated off from 10 cu cm, and it is certain that the implantation material did not contain more than 0·1 mg per cc. The same facts apply to all our experiments with ethereal extracts, including the digitonin precipitations, where the precipitate was an exceedingly small one, so small in some cases as to render impossible the recovery of the digitonin from the digitonide. Even the synthetic hydrocarbons were used at a concentration of 0·2%. Our interpretation is, therefore, that an implantation of maximal amounts of substances into the blastocoele cavity may bring about an induction either by injuring the ectoderm and so liberating the evocator already there, or by reason of the presence of natural evocator as an associated impurity. The second of these alternatives was clearly the case as far as glycogen was concerned, and we believe it to be the explanation of the results of Barth. The results of Fischer and his collaborators, on the other hand, we explain according to the first alternative.

If this view is correct, it might be possible, by treatment with various acids, to activate or unmask the evocator in isolated pieces of ventral ectoderm, and these should then induce on implantation. There is already a hint in the literature (Holtfreter, 1934, p. 275) that this can take place. Experiments along these lines will be carried out.

The argument of this section may therefore be summarized thus: the performance of an induction by implantation of a given substance may be brought about (i) because it contains an evocator as an impurity; (ii) because it is itself an evocator; or (iii) because it liberates a masked evocator within the tissue itself.

5—The Nature of the Inactive Evocator Complex

If we adopt the hypothesis that the evocator is a sterol-like substance, we can proceed to form a further working hypothesis as to the nature of the conditions which inactivate or mask it in the gastrula ectoderm. In this connexion, another line of work which has not so far been discussed in this paper is probably significant. For some time past the conviction has been growing among workers on this subject that the metabolism of carbohydrate, and especially glycogen, is intimately involved in organizer action. Although the claim of Fischer and Wehmeier (1933) that glycogen was itself the evocator was nullified by the finding of Waddington, Needham, Nowiński, and Lemberg (1935) that the ether-soluble active substance or substances are contained in ethereal extracts of crude glycogen preparations, the fact that crude glycogen may be effective is none the less of interest. Moreover, a special connexion of glycogen with the dorsal lip of the blastopore was reported by Woerdemann and his collaborators in a series of histochemical papers (Woerdemann, 1933; Raven, 1933). According to these authors, as invagination takes place through the blastopore lips, glycogen disappears from the cells. So sharp may be the boundary between the glycogen-rich ectoderm and the depleted mesoderm that Woerdemann spoke of a "*Glykogengrenze*". Woerdemann used the Carnoy-Langhans method; he was soon confirmed by Brachet (1934, *b*, p. 31) using the method of Bouin-Allen-Bauer, and by Jacobson (1935) using the method of Carnoy-Best. Tanaka (1934), using the Best-Kultschitzky method, could observe the boundary and the loss of glycogen on invagination, but saw it also at the ventral lip which does not induce so strongly as the dorsal lip. Finally, the position was rendered still more uncertain by the important finding of Pasteels and Léonard (1935) that if non-aqueous fixatives alone were used (dioxane) no

disappearance at all of glycogen on invagination could be detected, either on fish or amphibian material. Pasteels concludes that histochemical methods can hardly decide whether a disappearance of glycogen in the sections is a real metabolic phenomenon or simply an artifact due to the extraction of the glycogen from some cells and not from others during the processes of fixation and staining. It is true that even if the latter explanation held good for Woerdemann's results, they might indicate rather strongly a modification of the stability of the glycogen during invagination.

It was clear, then, that direct chemical analysis alone could settle the question of the disappearance of glycogen from the invaginated mesoderm during gastrulation. Mr. N. Heatley, one of our colleagues, undertook this investigation, using adaptations of the micro-chemical methods worked out by the school of Linderstrøm-Lang. His results, which will shortly be published in full elsewhere (Heatley, 1936), may be summarized as follows:—

LATE GASTRULA OF *Triton alpestris*

	mg glycogen / gm dry weight
Ventral ectoderm	160
Neural plate ectoderm	190
Invaginated archenteron roof mesoderm	110
Yolk endoderm	70

From this it clearly appears that Woerdemann's original view was partially correct, although based on inadequate reasons, and that there is a disappearance of some 40% of the ectodermal glycogen as invagination through the dorsal lip takes place. This is the first investigation in which it has been possible to make chemical analyses of morphologically distinct parts of the amphibian gastrula.

How does this loss of glycogen on invagination link up with the glycogen metabolism of the whole embryo during its development? Believing that the older researches with their unsatisfactory technique were not adequate for the needs of the present problem, a new investigation was made during the last laying season (Brachet and Needham, 1935). Besides estimating the total glycogen in the egg during development, following mainly the method of Good, Kramer, and Somogyi (1932), that portion of the glycogen bound in loose combination with proteins was also estimated. Willstätter and Rohdewald (1934) demonstrated that glycogen always exists in tissues in two forms, lyo-glycogen which is easily extract-

able with trichloracetic acid or boiling water, and desmo-glycogen which is combined with proteins.

The data may be summarized as shown in Table III. From these figures it is clear that there is a disappearance of some 30% of the initial glycogen of the egg during development. This is slightly more marked when the figures are expressed on a wet weight basis, since the weight of the whole system is rising owing to the imbibition of water (average 4·8 to 5·8 mg per egg; Needham, 1927), and slightly less so on a dry weight basis, since the total dry weight is, of course, falling (1·7 to 1·3 mg). Further, the bound or desmo-glycogen decreases from about 9% to 2½% of the total glycogen. These results are in agreement with those just published by Takamatsu (1936) for the tree-frog embryo (*Hynobius*).

TABLE III—EMBRYOS OF *Rana fusca*

Status	Time from fertilization hrs	Mg glycogen per embryo	Mg glycogen/gm Wet weight	Mg glycogen/gm Dry weight	% of glycogen as lyo-glycogen
Unfertilized	—	0·0802	16·7	47·2	91·1
Fertilized	0	0·0810	16·9	47·7	91·1
Young gastrulae ..	24	0·0812	16·4	49·4	93·2
Young neurulae	48	0·0760	15·0	47·5	95·1
Old neurulae	72	0·0672	13·1	44·8	97·0
Young tadpoles	96	0·0566	10·7	39·0	97·4

We have suggested above that the evocator, that is to say the substance which provides the direct stimulus to neural differentiation, is probably of an unsaponifiable, digitonin-precipitable nature, allied to the synthetic hydrocarbons used by Waddington and Needham. Further, we suggested that the acids and methylene blue, which also stimulate ectoderm to neural differentiation, act by unmasking this evocator substance, which had been present in an inactive form. This unmasking may also be brought about by any treatment which denatures the proteins, such as boiling or treatment with organic solvents (Holtfreter, 1933, *b*). There is thus a close analogy with the lipochrome astacin (Kuhn and Lederer, 1933; Kuhn, Lederer, and Deutsch, 1933; Fabre and Lederer, 1934), which, in combination with protein, makes the bluish-green pigment of crustacea, but which is liberated in its free red form by any treatment involving denaturation.

From all this it is permissible to make the tentative hypothesis that throughout the ectoderm and endoderm of the blastula there exists an evocator-glycogen-protein complex, analogous to desmo-glycogen,

lecitho-vitellin, or astacin. This complex breaks down wholly or partially only in the dorsal lip of the blastopore, liberating the active evocator.

In general there can be no doubt that such loose complexes exist. The evidence may be summarized from the literature as follows:—

Complexes of Proteins with Polysaccharides—Lyo- and desmo-glycogen (Willstätter and Rohdewald, 1934); myosin and glycogen (Przyłęcki and Majmin, 1934; polysaccharides and clupein (Przyłęcki, Gedroyć, and Rafałowska, 1935).

Complexes of Proteins with Unsaponifiable Lipins—Sterol-protein combinations in blood (Gardner and Gainsborough, 1927; Bruger, 1935; Mellander, 1935; Bills, 1935), in yeast (MacCorquodale, Steenbock, and Adkins, 1930), in wool (Schönheimer and Breusch, 1933); Astacin (Kuhn and Lederer, 1933); visual purple (Wald, 1935).

Complexes of Proteins with Saponifiable Lipins—Lecitho-vitellin (Fischer and Hooker, 1916); blood-lipins (Macheboeuf and Sandor, 1932; Wu and Chen, 1935; Delage, 1935; Grigaut, 1935); fat-protein complex in pancreatin (Schulman and Hughes, 1935).

Complexes of Saponifiable Lipins with Polysaccharides—Fatty acids and corn starch (Taylor and Nelson, 1920; Taylor and Sherman, 1933); lecithin and glycogen (Przyłęcki and Majmin, 1935).

Complexes of Unsaponifiable Lipins with Polysaccharides—Glycogen-evocator (Fischer and Wehmeier, 1933).

No need for hesitation exists, therefore, in postulating that the unmasking or activation of the evocator is really a liberation of it from inactive combination. We suggest that this is inherently much more likely than that a special inhibitory substance exists in the non-inducing parts of the egg, according to the hypothesis of Spemann, Fischer, and Wehmeier (1933).

Two processes therefore enter into consideration: (1) the splitting of the link between the proteins and the evocator-glycogen complex; (2) the disappearance of the glycogen itself, presumably in glycolysis, and the consequent freeing of the evocator. Since we know that evocator-glycogen complexes may be active, activation may imply only the first of these processes, but might involve both.

6—The Metabolism of the Organization Centre and the Action of Methylene Blue

From the experimental work reported in this paper, we see that the exposure of normally non-inducing tissue such as ventral ectoderm to the

action of a respiratory catalyst such as methylene blue results in the activation or unmasking of the evocator. Now the classical experiments on the sea-urchin of Barron and Hamburger (1932), Ellis (1933), and van Herk (1933) have shown that the respiration of some embryos is increased greatly by the addition of reversible oxidation-reduction indicators. One of us (J. N.) in collaboration with Mr. Feiwel and Mr. Claridge observed early in 1934 an increase in respiration of some 300% when $M \times 10^{-5}$ 4: 6-dinitro-*o*-cresol was added to eggs of *Echinus milearis*, and similar experiments were subsequently published by Clowes and Krahl (1934). This might at first sight lead to the conclusion that the inducing part of the egg has normally a higher respiratory rate than the non-inducing part, and that when by the action of a respiratory catalyst the respiration of the latter is raised to an abnormally high level, the evocator is activated or liberated from combination just as happens in the intact egg. This would fit in with the contention of Child based largely on Bellamy's old susceptibility experiments (Bellamy, 1919) that the dorsal lip of the blastopore is the dominant end of a " metabolic " gradient. Unfortunately, the second set of experiments here reported indicates that, as far as oxygen consumption is concerned, there is very little difference between the inducing and non-inducing parts of the gastrula, between the dorsal lip and the ventral ectoderm. There does, however, seem to be a well-marked difference in rates of carbon dioxide production between the dorsal lip and ventral ectoderm (Brachet, 1934, *b*) and the discrepancy between the results for carbon dioxide production and for oxygen uptake must now be discussed. The measurements of carbon dioxide production indicated a definitely increased respiration on the part of the dorsal lip: the oxygen uptake failed to establish any difference. The data cannot be quantitatively compared because the pieces of tissue in Brachet's experiments were not measured except as regards approximate equality of dimensions under the microscope. The ratio lip/ventral ectoderm, however, was 1·89–1·00. In the oxygen-uptake experiments, the ratio was 1·06/1·00. Now this discrepancy would be simply explained if the respiratory quotient of the dorsal lip was different from that of the ventral ectoderm. Since the larger quantity of CO_2 was given out by the dorsal lip, this would tend to have a higher respiratory quotient than the ventral ectoderm. Without, therefore, drawing any definite conclusions from these preliminary observations, and with the realization that it will be a matter of great technical difficulty to obtain respiratory quotients on such small material *in vitro*, we may be permitted the statement that a quotient of unity for the dorsal lip and of something less than that for the non-inducing parts of the egg is adumbrated by the data at present

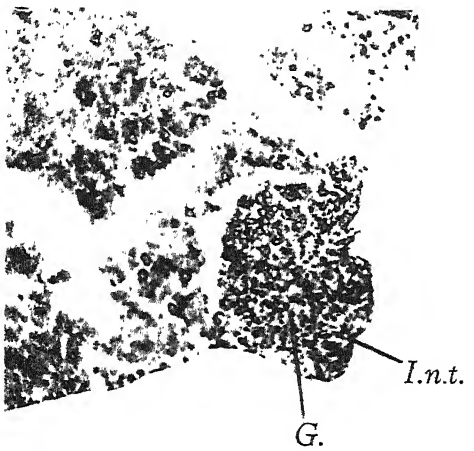

FIG. 2

FIG. 3

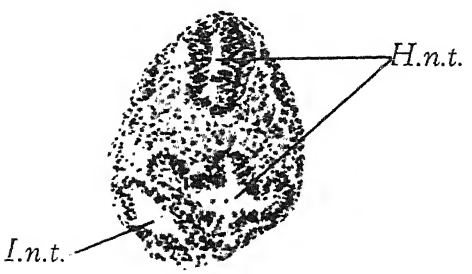

FIG. 4

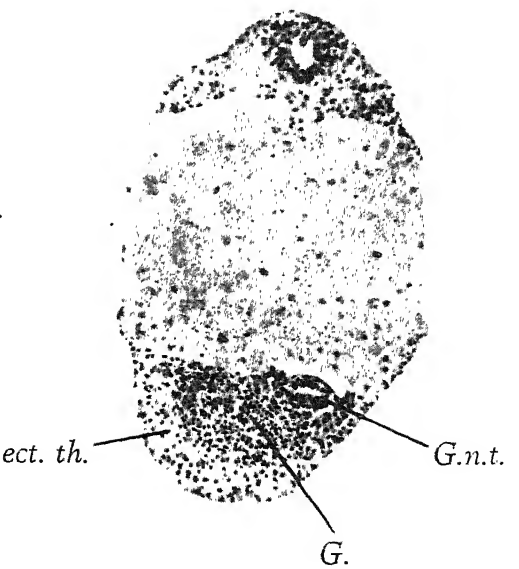

FIG. 5

existing. This would be of much interest in connexion with the finding of Brachet (1934, *a*) that the respiratory quotient of the whole amphibian egg is 0·7 (approximately) at the segmentation and blastula stages, rising during gastrulation to unity, and being maintained there during the rest of development. It may be therefore, that a predominantly carbohydrate oxidation begins at the dorsal lip during invagination and spreads out from there over the whole of the egg, and it may not be out of place to recall that in just such a manner the evocator is gradually liberated throughout the embryo after neuralation.

It may also be, however, that increased glycolysis in the dorsal lip leads to a liberation of CO_2 from carbonates there.

It is possible that the effect of methylene blue may be more closely connected with this difference in respiratory quotient than with a difference in respiratory rate. Experiments which have attempted to measure the effects of respiratory catalysts on amphibian eggs have not been very conclusive. Paraphenylene-diamine (Brachet, 1934, *a*) produces no rise in respiratory rate of intact amphibian eggs, and one of us (J. N.), with Dr. Nowiński, found in such experiments as were not rendered nugatory by the cytolysis of the eggs, only small increases with methylene blue and 4: 6-dinitro-*o*-cresol (up to 50%). Since the amphibian egg does not normally stain in the living state with methylene blue, it is probable that the surface is impermeable* to it, and this would account for the smallness of the effect. In any case, it is not necessary to postulate a large one. What is more important is its exact nature, and here it is significant that the action of methylene blue is believed to be exclusively upon carbohydrate catabolism. Barron and Harrop (1928) demonstrated this by showing that under its action a fall occurs in the glycolytic quotient of red blood corpuscles, and later Barron (1929) showed that inhibition of the preliminary stages of glycolysis inhibits also the effect of methylene blue. If this is so, then, we expose the ventral ectoderm, when we place it in a methylene blue solution, to a stimulus specific for carbohydrate catabolism, that is to say, a stimulus which must be similar to that which normally operates when first the dorsal lip of the blastopore, and then in due course the entire embryo, changes over to carbohydrate catabolism during gastrulation. It would be very interesting to know whether the isolated piece of ectoderm in the dye achieves a respiratory

* This impermeability seems to depend upon the intactness of the external cuticle, for when an extirpated piece of ectoderm is placed in the dye, it penetrates, at any rate partially. Other cases of one-way permeability are known, *cf.* the embryo kidney-tubules of Chambers and Kempton (1933).

quotient of unity in the two days before it is grafted into the blastocoele cavity of its designated host.

Besides this unmasking of the evocator by artificial alteration of the metabolism of the previously non-inducing ectodermal tissue, however, there are other possible interpretations. Evidence is brought forward by Nassonov and Alexandrov (1934) to show that the action of methylene blue on cells involves a reversible denaturation of part, at least, of the proteins. If this were so, the effects described in this paper would be due to the gentlest possible scission of the inactive evocator complex. But the whole subject of reversible denaturation is too obscure as yet to warrant more than a bare reference here to this possibility. It is interesting, however, to note that the dissolution and reformation of the lipochrome-protein complex of the eye (visual purple) is claimed by Mirsky (1936) as a case of reversible denaturation.

There might also be a direct effect of methylene blue on the sterol metabolism of the tissue. Roffo (1933) claims that in the plasma medium of tissue cultures there is a disappearance of cholesterol if methylene blue is present, and that the extent of this varies according to the concentration of the dye. It is greatest in the presence of growing chick heart-fibroblasts or of rat sarcoma, with 1/5000 methylene blue. Nothing is known of the mechanisms involved in this phenomenon.

So far, only the factors involved in the liberation of the evocator have been discussed. If this process could be inhibited without damage to the rest of the dorsal lip's metabolism, invagination would take place but no induction of neural structures in the overlying ectoderm. Such an effect seems to be produced when the dorsal lip is irradiated with ultra-violet light, for Dürken (1935) describes cases of invagination without induction under this treatment. In his discussion he envisages only the actual destruction of the evocator substance, and thinks it strange that a substance so stable to many chemical treatments should be so sensitive to ultra-violet rays. In view of the classical formation of vitamin D by irradiation of ergosterol, this effect is perhaps not so strange. But even more probably the radiation may be thought of as inhibiting the liberating processes. In the literature there is some contradiction regarding the action of ultra-violet rays on carbohydrate catabolism, for while Stiven (1930) found increased production of lactic acid in muscle extracts, a destructive effect upon amylase was observed by Kumanomido (1928) and Nadson and Stern (1934). Probably this may be resolved by the finding of Hutchinson and Ashton (1933) that shorter wave-lengths of ultra-violet destroy amylase, while longer wave-lengths stimulate it. We suggest, therefore (disregarding all questions of altered permeability),

that, in the experiments of Dürken, the liberation of the evocator from inactive combination may have been inhibited. A formal analogy to this is seen in the work of van d. Laan (1934) on auxin, the formation of which from its precursors by enzyme action is inhibited by ethylene.

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One of them (J. B.) was working in Cambridge as a travelling Fellow of the University of Brussels, and another (C. H. W.) held during the investigation a Senior Studentship of the Commissioners for the Exhibition of 1851.

7—Summary

The power of performing an induction can be caused to appear in those parts of the amphibian gastrula which do not normally possess it (such as the ventral ectoderm) by exposure to 100° C, or treatment with organic solvents. It is now shown that the same effect can be brought about by the action of a respiratory catalyst such as methylene blue upon the isolated ectoderm. Subsequent implantation of these isolated pieces next to competent tissue leads to the formation of a neural tube or to lesser degrees of neuralization, in the host or in the graft, or in both simultaneously.

This is interpreted as signifying the liberation of the natural evocator from previously inactive combination. The nature of this inactive combination is discussed and the suggestion is made that it is a complex of the type protein-glycogen-evocator. The present position of our knowledge of the metabolism of the organization centre is considered from this point of view.

A distinction is drawn between substances which cause neural inductions indirectly by liberating the masked evocator in active form, and those which cause neural inductions directly because of their identity with, or close chemical similarity to, the natural evocator itself. On the basis of an assessment of the minimal effective dose, it is suggested that nuclein compounds, and higher fatty acids, may belong to the former class, while active ether-soluble, unsaponifiable, digitonin-precipitable fractions belong to the latter class. A third type of substance, such as crude glycogen, and probably kephalin, act by virtue of the presence of substances of the second type in them as impurities.

Measurements of the oxygen-uptake of the dorsal lip of the blastopore and ventral ectoderm in a micro-respirometer indicate that only a very small, if any, difference in respiratory rate exists between these regions. The significance of this is discussed in relation to other findings on the metabolism of the gastrula.

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EXPLANATION OF PLATE 7

FIG. 2—CM–1. Implant of Axolotl ectoderm, kept 2 days in M $\times$ 10^{-4} methylene blue into Axolotl gastrula. Graft mass (G), induced neural plate (*I.n.t.*).

FIG. 3—C30*a*–2. Implant of Axolotl ectoderm, kept 2 days in M $\times$ 10^{-5} methylene blue, into *T. taeniatus* gastrula. Graft mass (G), induced neural tube (*I.n.t.*), and host neural tube (*H.n.t.*).

FIG. 4—C29*a*–4. Implant of Axolotl ectoderm, kept 2 days in M $\times$ 10^{-4} methylene blue, into *T. taeniatus* gastrula. Host neural tube (*H.n.t.*) and induced neural tube (*I.n.t.*).

FIG. 5—D34*b*–4. Implant of *T. alpestris* ectoderm, kept 2 days in M $\times$ 5 . 10^{-5} methylene blue, into *T. alpestris* gastrula. Graft mass (G.), containing a neural tube (*G.n.t.*) and ectodermal thickening (*ect. th.*).

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Studies on the Nature of the Amphibian Organization Centre

IV—Further Experiments on the Chemistry of the Evocator

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[PLATE 8]

INTRODUCTION

In preceding papers evidence was presented showing that a substance or substances able to bring about the differentiation of competent amphibian ectoderm into neural tissue is found in ethereal extracts of amphibian or mammalian liver. It was further shown that the active material could be traced into the unsaponifiable fraction and was precipitable with digitonin. The suggestion was therefore made that this substance, the evocator, was sterol-like in nature.

Further experiments in confirmation and extension of these findings are described in the present communication. During the laying season of 1935 we were much hampered by a diminished breeding activity of the newts owing to the very cold spring; and the quality of the eggs produced was poor, rendering them more suitable for experiments in which chemical preparations were not implanted (*cf.* Waddington, 1936; Waddington and Wolsky, 1936). However, a sufficiency of material was obtained to permit of the following up of three lines of attack: (1) the digitonin precipitation of ether-extracts of crude glycogen; (2) the fractionation of the total unsaponifiable material of liver; (3) the study of the activity possessed by crude kephalin preparations.

Chemical Technique

(1) *Digitonin Precipitation of Ether Extracts of Crude Glycogen*—Rabbits were starved for 48 hours then fed on carrots for three days; and the livers from freshly killed animals thoroughly ground. About 2 kg was used for each preparation.

To the tissue in a large flask twice its weight of 60% potash was added, and the flask then placed for three hours in a boiling water-bath under a reflux. After cooling, the mixture was diluted with twice its volume of distilled water, and an amount of 97% ethyl alcohol added corresponding to 2 cc for each cc of the diluted mixture. The copious precipitate of crude glycogen so produced was allowed to settle, filtered, washed twice with 66% alcohol (to which no salts had been added), then with 97% alcohol, and dried first in an oven at 37°, then in a calcium chloride dessicator. From 1964 gm liver 130 gm glycogen was obtained.

The crude glycogen was then extracted with ether in a Soxhlet apparatus provided throughout with ground joints, which had previously, together with the thimbles employed, been extracted with ether for from 7–17 hours. The extraction was carried out in an electrically-heated water-bath at a temperature between 45 and 55°, so that the thimbles were rapidly filled and emptied, and allowed to proceed for 140–170 hours. Every 48 hours, fresh ether was added and the former removed. The united extract was evaporated to dryness by distillation *in vacuo* at as low a temperature as possible. The solid residue was light yellowish-brown in colour, and weighed 164 mg.

This residue was then saponified as follows: it was taken up quantitatively in 6·5 cc of 2N KOH in 97% alcohol, and placed on a boiling water-bath under a reflux condenser for 2 hours. 1 cc 97% alcohol, 1 cc

distilled water, and 6·5 cc of 1·1N HCl were then added to the flask, making the resulting solution approximately 0·1N alkali.

This was then extracted with petrol-ether in a Dakin apparatus for 3½ hours, and the petrol-ether extract of unsaponifiable matter so obtained evaporated to dryness by gentle warming on a water-bath and dissolved in a small quantity of hot 97% ethyl alcohol. When a 1% solution of digitonin in alcohol was added to this, no obvious precipitation occurred but a slight cloudiness was produced. Nothing flocculated on allowing to stand in the ice-chest, but finally centrifugation was successful in bringing down the precipitate and clearing the supernatant fluid. The small precipitate was then washed with ether several times, and dissolved in a few drops of pyridine, aided by gentle warming. To this a little ether was added precipitating the free digitonin from the digitonides. After filtration the pyridine and ether were distilled off *in vacuo*, leaving a small residue of light yellowish appearance. This was dissolved in petrol-ether and kept in a container with ground joints, filled with nitrogen.

For implantation, it was added to coagulating egg-albumen, in the way previously described.

(2) *Fractionation of the Total Unsaponifiable Material of Liver*—Our starting-point here was the work of the Liverpool school on the hydrocarbons of pig liver, described by Channon, Devine, and Loach (1934). We were provided with actual samples of two of their fractions. Starting with their total unsaponifiable material, they first threw out a large quantity of sterols by crystallization from light petroleum (b.p. 40–60°) at room temperature. This will be called in the present discussion " Channon's Fraction I " (*see* p. 2017 of their paper). They next crystallized a further quantity of sterols from acetone at 0° or under; this fraction (" Fraction II ") has not been tested by us. The remaining sterols they precipitated with digitonin, which was subsequently recovered in the following way (H. J. C.): the Windaus method (1910) was first tried and found unsatisfactory, and the thimble containing the digitonides then left standing some months in xylene. The digitonides were then mixed with the xylene and the whole evaporated to dryness *in vacuo* on a water-bath. The dry residue was then extracted with 90% alcohol to remove any digitonin and cholesterol liberated by the original xylene treatment; this alcoholic extract was evaporated to dryness and extracted with ether. The cholesterol was recovered by evaporation of the ether. The original bulk of the digitonides which had not been decomposed by the original treatment with xylene was then dissolved in pyridine and the solution treated with ten volumes of ether. The precipitated digitonin

was removed by filtration, the pyridine-ether mother-liquor was then transferred to a separating-funnel and shaken with dilute hydrochloric acid many times to remove the pyridine. When the ethereal solution was pyridine-free, it was washed to neutrality with water and evaporated. The cholesterol (and other sterols, if present) was added to that previously mentioned. This we describe as " Fraction III ".

It is hoped to test the hydrocarbon fractions obtained by Channon, Devine, and Loach, but in view of the previous indications on the chemical properties of the evocator, attention has so far been concentrated on the first crop of sterol crystals and the digitonin-precipitable fraction.

Our sample of Fraction I was tested as such, but further treatment was applied to Fraction III. This was reprecipitated with digitonin and the digitonin recovered by the Schönheimer-Dam method (1933). The residue was taken up in a little hot methyl alcohol; the insoluble portion was not further followed, but the soluble part was subjected to fractional crystallization. By alternate cooling, evaporation, cooling, and further evaporation, a series of crystal-crops was separated, which we refer to as Fraction III A1, A2, A3, A4, A5.

During the course of the present work, there appeared the paper of Fischer, Wehmeier, Lehmann, Jühling, and Hultzsch (1935), in which the suggestion is made that a variety of substances may act as evocators by providing an acid stimulus (" Säure-Reiz"). In order to test the possibility that our active Fraction I was acting in this way, we treated it in the following manner. 2 gm were dissolved in 70 cc of ether and shaken three times with aqueous decinormal soda. The ethereal solution was then washed three times with distilled water. Some sterol-like substances had evidently passed out into the aqueous soda, whether by way of emulsification or otherwise, for the aqueous extract referred to above showed a positive Liebermann-Burchard reaction. The alkaline extract was therefore shaken many times with ether after being nearly but not quite neutralized with hydrochloric acid. On crystallization of this ether extract a crop of pink sterol-like crystals was obtained from this ethereal fraction. We refer to these fractions as follows:—acid aqueous after the first ether extraction α; sterol β; final aqueous fraction γ.

(3) *Preparation and Treatment of Kephalin*—Barth (1934) chose for his method of preparation of kephalin a rather unusual procedure; the minced brain was extracted with ether, and then by means of successive precipitations with alcohol and acetone, he obtained a preparation which was admittedly not very pure. It seemed important to test the possibility that the evocator activity possessed by Barth's preparations was really due to the admixture of small amounts of sterol-like substances.

We therefore made use of three different methods of preparing kephalin: that of Barth, that of Parnas and Renall (Renall, 1913), and that of Page and Bülow (1931). The difference between the first two of these methods is that Barth extracts the brain directly with ether and then subsequently attempts to remove the sterols, while in the Parnas-Renall method, the minced brain is first of all treated with acetone, which extracts the main mass of the sterols, before the ether extractions are begun.

The third method, that of Page and Bülow, is quite different from the preceding ones, for both of them lead to a preparation which is alcohol-precipitable. In contrast, it was shown by Rudy and Page (1930) that kephalin precipitated by alcohol is unsatisfactory in that it gives low carbon figures and low iodine values. On the other hand, the highly unsaturated kephalin which remains with lecithin in alcoholic solution is believed to be more similar to the natural product. Separation from the lecithin is effected by the barium salt method. The Page-Bülow method has, furthermore, the advantage that the first stage of the process consists in a long-continued extraction of the minced brain with acetone. This is carried on until the extract shows a negative Liebermann-Burchard reaction—an operation which may take as long as a week. We are therefore certain that the tissue to be treated with petrol-ether contains only the slightest traces of sterols.

If kephalin is not itself active as an evocator, but some unsaponifiable substance or substances associated with it, then the destruction of the kephalin by saponification should leave the evocator activity undestroyed. Saponification of the kephalin was carried out as follows. After evaporation from petrol-ether to dryness, the preparation was boiled under a reflux condenser for 10–36 hours with 20 cc of 2N alcoholic KOH. A portion of the kephalin remained undissolved, and at the end of the saponification was removed by filtration. To the filtrate was added N/HCl in the proportion HCl: KOH 1·5: 1·0, and the alcohol concentration reduced to 50%. The unsaponifiable matter was then separated by continuous extraction in a Dakin apparatus with petrol-ether (b.p. less than 40°). The unsaponifiable fraction gave a strongly positive Liebermann-Burchard reaction.

Description of Specimens

All the implantations were made in coagulated egg-albumen, as described previously (Waddington, Needham, Nowiński, and Lemberg, 1935), except where otherwise stated.

D46*b*–6. Digitonin precipitate of unsaponifiable fraction of ether extract of glycogen.

Triton alpestris—The embryo was fixed on the fourth day after operation, in the middle tail-bud stage. A large lump was visible on the belly. The sections show an induced neural tube within this lump. The lumen is narrow and contorted and the walls of the tube are thin, in most places only consisting of one layer of cells, but the histological differentiation is perfectly typical; fig. 1, Plate 8.

D82*b*–11. "Sterol β Fraction" (*see* p. 201).

Triton alpestris—Two days after the operation the host embryo was in the open neural plate stage. An induced neural plate could be seen, joined to the anterior region of the host plate and running away at right angles from it towards the implant. The part of the induced plate which covered the implant was considerably wider than the part nearest the host plate. Since the batch of eggs were not very healthy, the embryo was fixed. The section is perpendicular to the host axis and cuts the induced plate longitudinally; fig. 2, Plate 8.

D22*b*–2. Channon's Fraction I.

Triton alpestris—The embryo was fixed on the sixth day after the implantation, when the first contractions were beginning. A large ectodermal thickening was present on the belly, within which the sections reveal the presence of an induced neural tube. The walls are thin, and the nuclei not so elongated as might be expected, but there can be no doubt of the neural nature of the induced structure. Owing to the bending of the embryo in the embedding, the sections do not cut the tube in the most favourable direction, fig. 3, Plate 8.

The substance to be implanted was not emulsified in coagulated albumen in the way previously described, but was taken up in a mixture of uncoagulated albumen and sesame oil, of the consistency of butter. Controls (D33*b*) showed this material to be inactive.

D18*a*–20. Acetone extract of brain (Page and Bülow).

Triton taeniatus—The implantation was made in a mixture of uncoagulated albumen and sesame oil. Two days after the operation, the embryo was in the open neural plate stage, with a clearly recognizable induced neural plate on the right side of the belly. On the fourth day it was still in a stage with just closed neural plate, and was then fixed. In the sections, the host neural plate is just closed, but not yet free from the surface, while the induced plate is still open. The histological differentiation of the induced plate is typical but it is thinner than a normal neural plate.

D65*b*–6. Unsaponifiable fraction of kephalin.

Triton alpestris—The embryo was fixed in the middle tail-bud stage on the fourth day after the operation. There is a large lump on the belly, mainly filled by the implanted mass. The cells of the inner layer of the ectoderm covering the implant are elongated, and have oval nuclei arranged in a palisade. In some places the epithelium is quite thin and the tissue is very obviously neural in nature. The thickening as a whole is classified as B+++; fig. 4, Plate 8.

Discussion

From the specimen just described, and from the first portion of the accompanying statistical table (Table I) it can be seen that neural inductions are obtainable with the digitonin precipitated unsaponifiable material of an ether extract of crude glycogen prepared from mammalian liver. In series C88*d* it was assumed that a sufficient saponification of saponifiable substances would take place in the boiling potash of the Pflüger method, but in order to ensure this more fully, the ether extract of the glycogen was itself saponified in the later work (series D35*b*, 36*b*, and 46*b*) as described in an earlier section of this paper.

Next to be considered is the unsaponifiable matter of pig liver (Channon, Devine, and Loach, 1934). From Table I it will be seen that most of the activity is in Fraction I, *i.e.*, the sterol mixture which crystallized most readily from light petroleum at room temperature. This being the case, it is not surprising that Fraction III, which was separated later in the same fractionation, shows little activity. In connexion with the "acid-stimulus" theory of Fischer and his collaborators, it is important that after washing Fraction I (prep. 73) with aqueous soda to remove acidic substances, the material (prep. *p*73) lost very little of its activity. That the aqueous extract so obtained also showed some activity is explained by the fact that by further treatment with ether it was possible to isolate a fraction, which we describe as "Sterol β", which had entered the aqueous layer either by way of emulsification or because possessing a lyophil or phenolic structure. The final aqueous extract, so far as it was tested, was inactive. These experiments, therefore, give no support to the suggestion of Fischer *et al.* (1935) that positive findings of evocator activity in unsaponifiable ethereal fractions can be explained by the presence of acidic substances in them.

With regard to the question of kephalin, it can be seen from Table I that few implantations of this substance were made, and that, of these, not many embryos came through to sectioning. This, however, was not

TABLE I—EXPERIMENTAL RESULTS

Fraction tested	Serial prep. No.	Series	No. embryos cut	A	C	B+++	B++	B+	B−	D	E	I	S
(1) Glycogen extracts:													
Digit. ppt. ether ext. liver-glycogen:													
(W. W. N.)*	—	C88*d*	6	1			1		1	1	2		
(A. C.)	66	D35*b*, 36*b*, and 46*b*	14	1			2	1	2	2	5		
(2) Unsaponifiable of liver:													
Channon Fraction I	73	C95*d*, D22*b*, 23*b*, 28*d*, 53*b*	36	4		2	2	4	8	9	1	5	1
Do., shaken with soda	*p*73	D71*b*, 72*b*, 86*b*, 88*b*, 90*b*	23	1		2	3	4			9	4	
Aqueous extract	α	D74*b*	5			2				2	1		
"Sterol β"	β	D82*b*, 87*b*	19	2†		2	1		3		8	2	1
Final aqueous extr.	γ	D92*b*, 93*b*	5						2	1	2		
Channon Fraction III:													
A1 and A2	—	D49*b*, 61*b*	8				2			3	3		
A3	—	D48*b*, 51*b*	10							5	2	3	
A5	—	D63*b*	12				2			3	5	2	
(3) Kephalin:													
Acetone extract of brain (Page-Bülow)	54	D2*d*, 4*d*, 9*a*, 11*a*, 13*a*, 16*b*, 18*a*	24	2		1	2	1	1	6	2	9	
Kephalin (Barth)	59*a*	D12*a*	1								1		
Kephalin (Parnas)	63*a*	D50*b*	4							3	1		
Saponifiable fraction of kephalin (Barth)	57	D24*b*, 32*b*	10				1	5	2	1	1		
Unsaponifiable fraction of kephalin (Barth)	58	D25*b*, 31*b*, D65*b*	20			2		1		5	9	3	
(4) Controls:													
Egg-albumen	—	C87*d*, 96*d*, D33*b*	20					1	7	12			

* This series was mentioned already in the first paper (Waddington, Needham, Nowiński, and Lemberg, 1935), but is included here for the sake of completeness.

† This figure includes one induced blastopore.

Key—*a*, *Triton taeniatus*; *b*, *Triton alpestris*; *d*, *Amblystoma*. Classification according to conventions of first paper (1935). A, C, B+++, B++, positive; B+ doubtful; the others negative.

important; we were not concerned to test the evocator activity of kephalin preparations, since we fully accept the results of Barth (1934). The significant observation now made was that the acetone extract of brain, made according to the Page-Bülow method, and containing no kephalin, possessed considerable activity. Furthermore, after saponification of kephalin prepared according to Barth, activity was still present, mainly in the unsaponifiable fraction. The chemical procedure, already described, was certainly sufficient to break down the kephalin completely, and the persistence of activity can only be ascribed to the presence of small amounts of other substances stable to saponification. Taken together with the activity of the sterol-containing acetone extract of brain, the results on the unsaponifiable fraction of liver, and the activity of digitonin-precipitable material extracted from glycogen, this result justifies us in assuming that the active substance accompanying kephalin preparations is of a sterol-like nature. The difficulty of removing sterols completely from brain tissue, and from phosphatide preparations, is in any case well known.

Finally, Table I contains details of further control experiments with egg-albumen, which was the implantation medium used throughout. In the four papers of this series (excluding the methylene blue experiments, which stand by themselves) a total of 478 embryos have been sectioned, and of these 308 have belonged to series in which positive results were obtained. The total number of controls amounts to 83, uniformly negative, *i.e.*, 17·4% of the former figure, and 26·9% of the latter.

The authors wish first to express their gratitude to Professor H. J. Channon for his kind assistance, especially in the provision of samples from his large-scale fractionation of pig liver sterols and hydrocarbons. They also offer their warmest thanks to Frl. J. Kriebel, whose histological ability greatly aided the work. One of them (W. W. N.) held a Rockefeller Travelling Fellowship during the course of the experiments. Another (R. L.) is indebted to the Academic Assistance Council, and a third (A. C.) was the holder of a Moyse Travelling Scholarship from McGill University, Montreal. Finally, they wish to thank the Government Grant Committee of the Royal Society for financial support.

Summary

The presence of an evocator substance in the digitonin precipitate of the unsaponifiable material of ethereal extracts of crude glycogen is confirmed.

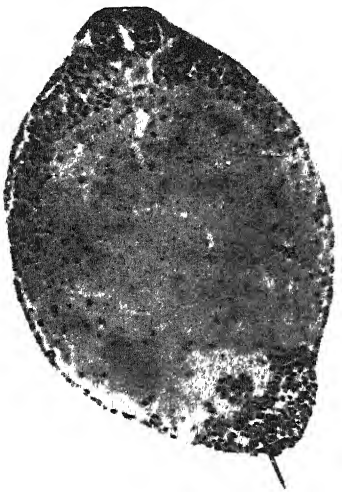

FIG. 1

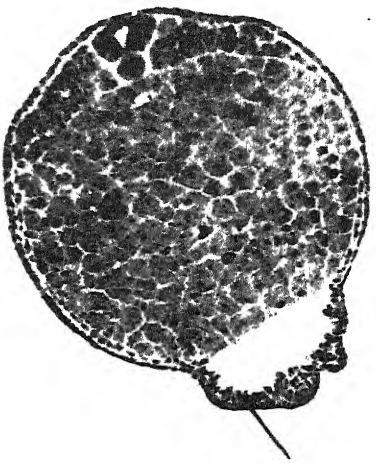

FIG. 4

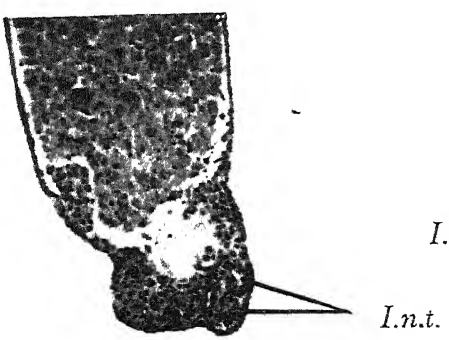

FIG. 3

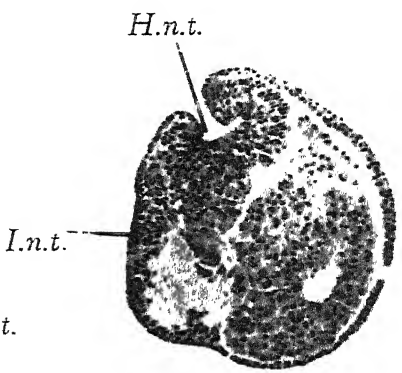

FIG. 2

Attempts at fractionation of the sterolic portions of the unsaponifiable material of adult mammalian liver are described.

It is shown that acetone extracts of adult mammalian brain, which should contain no kephalin, may be active, and that the activity of kephalin preparations persists after the destruction of this lipin by saponification.

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Explanation of Plate 8.

Fig. 1—D46*b*–6. Digitonin precipitate of unsaponifiable fraction of ether extract of glycogen.

Fig. 2—D82*b*–11. Sterol β fraction.

Fig. 3—D22*b*–2. Channon's Fraction I.

Fig. 4—D65*b*–6. Unsaponifiable fraction of kephalin. *I.n.t.*, induced neural plate or tube; *H.n.t.*, host neural tube; *ect. th.*, ectodermal thickening, grade B+++.

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Competition Between Bivalents During Chiasma Formation

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INTRODUCTION

It has long been known that crossing over in one section of a chromosome reduces the probability of further crossing over in other sections of that same chromosome. More recently it has been demonstrated by Haldane (1931) that the frequency distributions of chiasma formation in bivalents at the first division of meiosis also indicate that interference occurs.

It has, however, been assumed that crossing over in one bivalent is independent of crossing over in other bivalents of the same nucleus. Recent work has rendered doubtful the validity of this assumption.

Genetically it was found that prevention of crossing over in two of the long chromosomes of *Drosophila melanogaster* resulted in increased crossing over in the third chromosome (Morgan, Bridges, and Schultz, 1933). De Winton and Haldane (1935) have, however, failed to find similar correlation of crossing over in normal chromosomes of *Primula sinensis* and mention like failure with *Pisum sativum*. Hence it is not clear from the available genetical data whether the apparent negative correlation of crossing over in *Drosophila* is due to a generally occurring effect of this kind or to the peculiar circumstances arising as a result of the use of inversions to prevent crossing over in two of the three long chromosomes.

Cytological data bearing on this question are also available and are more valuable as they can be acquired from the study of a larger range of organisms than is possible genetically. Darlington (1933) gives data on the frequency of chiasma formation, *i.e.*, crossing over, in the seven long and single short bivalent of eight chromosome rye. He constructed a correlation table having as the two variates the total number of chiasmata in the seven long bivalents and in the short one from the same nuclei. Instead of finding a positive correlation of these two chiasma frequencies, as he expected from a consideration of environmental effects on chiasmata, he obtained a negative correlation. This clearly showed an effect of

chiasma formation in one bivalent on chiasma formation in others of the same nucleus.

Sax (1935), analysing chiasma frequency data from *Vicia faba*, found the chiasma frequency of the M bivalent to be uncorrelated with the total chiasmata of the five m bivalents. On the basis of this result he criticized Darlington's findings and doubted the existence of the phenomenon. However, Mather and Lamm (1935) confirmed the results of both workers. They found that normal seven chromosome rye showed a negative intra-class correlation within the seven chromosomes, and that, in *Vicia*, while the M bivalent showed a chiasma frequency distribution independent of the total of the five m bivalents, the latter showed a negative correlation among themselves.

Thus there can be little doubt that the phenomenon is real but is not universal. The occurrence of the negative correlation is not even consistent within the same nuclei when different groups of bivalents are concerned. The present work was undertaken with a view to clarifying this very obscure situation, by examining the incidence of the phenomenon over as wide a range of material as could be obtained and by attempting to obtain some idea of the mechanism behind the results.

The method of correlations was used exclusively. A positive correlation of the chiasma frequencies of two or more bivalents may be explained as a result of environmental influences or mixed material since such factors must cause the deviations of all the bivalents in the same nucleus to tend in the same direction. A negative correlation must indicate some influence of one bivalent on chiasma formation in others of the same nucleus irrespective of outside influence. This phenomenon will be referred to here as "competition". The reason for the choice of this name will be found in the discussion of the mechanism behind the results. The negatively correlated chiasma frequencies are just the signs of competition which could perhaps be detected in other ways, although no other method is as yet apparent. Bivalents whose chiasma frequencies are correlated will be described as showing competition, or as competing, those not correlated, as not competing.

As will be seen from the next section, the material included 27 species and hybrids covering both insects and flowering plants, and including in the latter both Dicotyledons (3 genera) and Monocotyledons (6 genera).

I wish to acknowledge my indebtedness to the following for allowing me to use either unpublished data or preparations of meiosis in various organisms: Dr. A. Levan, Hilleshög, Sweden, and Dr. A. A. Moffett, Miss M. B. Upcott, and Mrs. E. R. Sansome, of the John Innes Horti-

cultural Institution. I am also indebted to Professor R. A. Fisher for advice with respect to the statistical treatment and interpretation of the data.

Material and Methods

The frequencies of chiasma formation in the following organisms were used: *Culex pipiens*, *Crocus biflorus*, *C. Kowolkowii*, *Eremurus* sp., *Kniphofia Nelsonii*, *K. Burchelli*, *K. leicthlinii*, *K.* "*corallina*", *Pisum sativum*, *Vicia faba*, *Oenothera* sp., *Allium macranthum*, *A. zebdanense*, *Secale cereale*, *Locusta migratoria*, and *Tulipa* var. "Pink Beauty". Only completely analysable nuclei were used. The necessity for obtaining such nuclei limited the range of material.

This material was chosen to satisfy two criteria as far as possible. First, the chiasma frequency as observed at metaphase should not differ greatly from the actual frequency of chiasma formation. Since terminalization is the only process that would result in such a difference the material was chosen to have low terminalization. Wherever available the metaphase chiasma frequencies were compared with diplotene chiasma frequencies. If this was not possible the material was chosen as having a low terminalization coefficient. There is one exception to this rule. The *Oenothera* results are from plants with complete terminalization. However, the only effect of this would appear to be a "damping" of the correlation. This case is more fully discussed in the text.

The second criterion was that size differences between the chromosomes should not introduce statistical complications. This will be understood better after a discussion of the statistical methods.

As pointed out by Mather and Lamm (1935) there are two methods available for the detection of correlations in such data. The first is by the construction of a correlation table showing the simultaneous distribution of the chiasma frequencies of the two classes of configurations. The correlation coefficient is then calculated from the formula

$$\frac{S(xy)}{\sqrt{S(x)^2 \,.\, S(y)^2}}$$

where $S(x)^2$ and $S(y)^2$ are the sums of squares of the deviations of the individual distributions from their means and $S(xy)$ is the sum of products of the simultaneous deviations of each observation from the two means. This process is well known and is fully described in any textbook on statistics.

This first method is suitable only for cases where two classes of configurations can be consistently distinguished, as for example by length differences, *e.g.*, *Vicia faba* with long M and short m bivalents, or by some peculiarity of structure such as ring formation in *Pisum* and *Oenothera*.

The second method which is of more general use, is the use of the analysis of variance to detect intra-class correlations. Intra-class correlation coefficients are not given, as the detection of correlation is the important point. Suppose we have observations on the chiasma frequencies of k bivalents over a range of n nuclei. The analysis of variance enables us to divide up the variation of the joint distribution of these bivalents into three parts. The general principle of this is easily shown.

We may set down the observed chiasma frequencies of the k bivalents in the first nucleus as $a_1, b_1, c_1, \ldots, k_1$, and so on for the n nuclei, thus:—

a_1	b_1	c_1	.....	k_1	m_1
a_2	b_2	c_2	.....	k_2	m_2
a_3	b_3	c_3	.....	k_3	m_3
..	..	..		..	..
..	..	..		..	..
..	..	..		..	..
a_n	b_n	c_n	.....	k_n	m_n
m_a	m_b	m_c	.....	m_k	

The upright margin gives the mean chiasma frequency of the bivalents in the various nuclei ($m_1 \ldots m_n$). The horizontal margin gives the mean chiasma frequency of the different bivalents over the whole range of nuclei ($m_a \ldots m_k$).

We may then recognize the first part of the variance of the complete distribution as being ascribable to differences between the means of the different bivalents as calculated from the horizontal marginal totals. This will be termed the mean square or variance between configurations.

The second portion of the variation corresponds to, and is calculated from, the differences between the nuclear means as shown in the upright margin. This will be termed the between nuclei mean square or variance.

If the marginal totals are given it is possible to formulate an expected chiasma frequency for all the bivalents in each nucleus and the third portion of the variance is ascribable to the deviations of the observed

values from these expectations. This represents the variation of the bivalents after allowance has been made for differences between both configurations and nuclei and so may be termed the inherent variance or mean square. The inherent mean square is dependent on interference in chiasma formation within the bivalents.

If there is no correlation between the chiasma frequencies of the different bivalents over any set of nuclei, it is possible to show that the mean square between nuclei should be the same as the inherent mean square. If there is a positive correlation of the chiasma frequencies of the bivalents, their deviations from the appropriate means will tend to be in the same direction in any nucleus, and then the between-nuclei mean square will be greater than the inherent variance. On the other hand, with a negative correlation the deviations of the bivalents will tend to be compensatory and the variance between nuclei will be less than the inherent variance. The significance of the difference between any two mean squares may be tested by the use of Fisher's z distribution.

There often arises a complication in the analysis of variance as applied to the present material. It is not always possible to make consistent distinctions between all the bivalents over any range of nuclei. It is clear that, in such cases, the lower marginal totals cannot be obtained, and so the between-configurations mean square cannot be separated from the inherent mean square. In such cases the separation of the variance is into two parts only, the mean square between nuclei and the remainder, termed herein the mean square within nuclei. This latter is a compound of the inherent and the between-configurations mean squares. Now it is clear that if the differences between the mean chiasma frequencies of the bivalents concerned are great the contribution of the configuration mean square will be large. This may lead to the within nuclei mean square being such that there is an apparent negative correlation when the use of the inherent variance would give no such indication.

But it is known that, apart from very short bivalents, the mean chiasma frequencies of the bivalents, in any organism, are approximately proportional to their lengths. Hence there exists a check on any negative correlations detected by the incomplete analysis of variance into mean squares within and between nuclei. Wherever this incomplete analysis had to be used the negative correlation was tested with a view to determining whether it could be explained by the known size range of the chromosomes in that particular organism.

The reason for the second criterion is now obvious. The size differences of the chromosomes must be such that either the bivalents can be regularly distinguished, in which case either the correlation table method or the

TABLE I*

Species	n	No. of nuclei	Comparison x v. y	Mean x	Mean y	Sum of squares and cross-products S (x^2)	S (xy)	S (y^2)	Mean squares Within nuclei	Mean squares Between nuclei	Significance %
Culex pipiens 1	3	25	2 long v. 1 short	1·08	1·00	3·3600	0	0			
,, 2	3	42	,,	1·19	1·02	11·7561	0·6098	0·9756			
,, 3	3	8	,,	1·25	1·00	4·0000	0	0			
,, 4	3	17	,,	1·50	1·31	6·0000	1·0000	2·7992			
,, 5	3	18	,,	1·68	1·29	7·2143	0·4286	2·8571			
,, 6	3	19	,,	1·79	1·34	5·4286	2·1429	3·2143			5
Crocus biflorus	4	101	2 long v. 2 short	2·52	1·61	82·4208	13·7822	80·1000			
,, ...	4	101	within 2 long	2·52					0·3713	0·4492	
,, ...	4	101	within 2 short	1·61					0·3713	0·4260	
C. Kowolkowii	10	25	2 long v. 8 short	2·98	1·55	41·7600	13·3640	26·2384			5
,, ...	10	25	within 2 long	2·98					0·5400	0·5617	
,, ...	10	25	within 8 short	1·55					0·2175	0·2875	
Eremurus sp. 1	7	40	5 long v. 2 short	2·87	1·61	191·96	5·95	18·97			
,, 2	7	19	,,	2·80	1·58	94·53	8·43	6·53			
,, 3	7	36	,,	2·80	1·64	76·00	6·00	11·22			
Kniphofia Nelsonii	6	20	within 6 bivs.	1·84					0·4450	0·3943	
,, *Burchelli*	6	22	,,	1·61					0·3061	0·2785	
Pisum sativum	7	50	ring of 4 v. 5 bivs.	5·08	2·52	75·775	0·725	28·775			
,, ...	7	50	within 5 bivs.	2·52					0·5550	0·4134	
Locusta migratoria	11	70	2 long v. 6 medium	2·15	1·13	24·2857	9·2857	47·7857			2
,, ...	11	70	within 2 long	2·15					0·2357	0·1935	

* Where an inter-class correlation is used, the table gives the mean of each of the two classified types, the two sums of squares and the sum of cross-products. Where the analysis of variance is used the table gives the general mean, and the mean squares within and between nuclei. The actual value of r, the inter-class correlation coefficient is not given, as it is detection, not estimation, which is important. The significance column gives the probability of obtaining such a result from an uncorrelated population.

TABLE II*

Species	n	No. of nuclei	Comparison x v. y	Mean $\bar{x}$	Mean $\bar{y}$	Sum of squares and cross-products S (x^2)	S (xy)	S (y^2)	Mean squares Within nuclei	Mean squares Between nuclei	Significance %
Kniphofia Leicthlenii	6	20	within 6 bivs.	1·70	—	—	—	—	0·4367	0·1860	5 <> 1
„ "*corallina*"	6	26	„	1·44	—	—	—	—	0·3462	0·1344	1
Vicia faba	6	50	1 long v. 5 short	7·06	3·42	86·50	−6·30	62·82	—	—	—
„	6	50	within 5 short	3·42	—	—	—	—	0·7080	0·3531	1 <
Oenothera—											
blandina-parviflorens ..	7	35	(12) v. (2)†	—	—	58·97	0·17	4·97	—	—	See in text
serratens-rubricalyx α	7	25	—	—	—	24·24	−1·36	5·04	—	—	
deserens-nutens	7	42	(10) v. 2(2)	—	—	31·33	−1·67	1·90	—	—	
grandiflorens-divergens	7	27	—	—	—	26·96	0·89	4·67	—	—	
serratens-purpurata ..	7	17	(8) v. 3(2)	—	—	7·53	−1·12	2·35	—	—	
dependens-purpurata ..	7	38	—	—	—	39·71	−2·74	3·58	—	—	
deserens-blandina ..	7	79	(6) v. 4(2)	—	—	32·61	−2·29	10·73	—	—	
blandina-purpurata	7	34	(4) v. 5(2)	—	—	6·26	−0·74	4·26	—	—	
purpurata-blandina	7	30	—	—	—	1·87	−0·13	1·87	—	—	
deserens-purpurata ..	7	44	—	—	—	12·25	−0·75	2·80	—	—	
dependens-blandina ..	7	41	(8) v. (4) v. (2)	—	—	—	—	—	0·3043‡	0·2659	
serratens-blandina	7	38	—	—	—	—	—	—	0·3945‡	0·2686	

										1·2792	0·3304	5
Allium macranthum	14	8	within 6 long	4·60	—	—	—	—		0·5417	0·4107	—
,, ...	14	8	within 4 medium	3·06	—	—	—	—		0·3021	0·3527	—
,, ...	14	8	within 4 short	1·41	—	—	—	—		0·4213‡	0·4162	—
Allium zebdanense 1	9	20	within bivs. I-V	2·56	—	—	—	—		0·2228‡	0·0684	1
			within bivs. VI-IX	1·30	—	—	—	—		0·4152†	0·8849	
,, 2	9	12	I-V	2·93	—	—	—	—		0·1420‡	0·1117	—
			VI-IX	1·48	—	—	—	—		0·2256‡	0·1422	—
,, 3	9	10	I-V	2·48	—	—	—	—		0·2037‡	0·1222	—
			VI-IX	1·35	—	—	—	—		0·3633‡	0·5689	—
,, 4	9	10	I-V	2·56	—	—	—	—		0·1777‡	0·2888	—
			VI-IX	1·40	—	—	—	—		1·2017	0·2128	1
Tulipa " Pink Beauty "	12	8	within 12 trivs.	3·20	—	—	—	—				

* Where an inter-class correlation is used, the table gives the mean of each of the two classified types, the two sums of squares and the sum of cross-products. Where the analysis of variance is used the table gives the general mean, and the mean squares within and between nuclei. The actual value of r, the inter-class correlation coefficient is not given, as it is detection, not estimation, which is important. The significance column gives the probability of obtaining such a result from an uncorrelated population.

† (12) = ring of 12 chromosomes as maximum configuration, (2) ring bivalent, etc.

‡ Inherent mean square.

complete analysis of variance may be used, or they should be small enough not to invalidate the use of the incomplete analysis of variance.

The arithmetic procedure of the analysis of variance is described by Fisher (1934) and reference should be made to that publication for details of the method.

Observations

Summaries of the different sets of observations are given in Tables I–III. Where an inter-class correlation was used, the tables give the sums of squares and of cross-products. Where the analysis of variance was used the mean square between nuclei and either that within nuclei, or the inherent mean square are given. Correlation coefficients are not given as it is the significance, not the magnitude, which is of importance. Some of the observations may be dismissed immediately as providing nothing but negative evidence. These are the results given in Table I. In none of the species listed in that table is there evidence for competitive chiasma formation. There is a suggestion of positive correlation in some cases but this, of course, may be interpreted as being a result of mixed data, *i.e.*, to an effect of environment on chiasma formation in these organisms. It will be noticed that in general the species listed in this table have a low number of chromosomes or a low chiasma frequency.

The remainder of the observations are more informative and will be dealt with in some detail. The observations on *Secale* are summarized in Table III and the rest in Table II.

Oenothera sp. (Catcheside, 1933)—Of the 12 analyses, 10 were made by the inter-class correlation method. The configurations were grouped into the ring of chromosomes and those outside the ring. The chiasma frequencies were calculated from the detailed frequencies of rings and chains of various numbers of chromosomes given by Catcheside, since with complete terminalization a ring of two chromosomes has two chiasmata, a chain of two one chiasma and so on. In Table II are given the sums of squares and of cross-products of the chiasma frequencies of the two groups of configurations. The correlation coefficient is given by the formula

$$r = \frac{S(xy)}{\sqrt{S(x)^2 . S(y)^2}}.$$

Of these 10 cases none give significant correlation coefficients. There are, however, 8 with negative correlations and 2 with positive ones. This would suggest that although there is no single significant negative

TABLE III—*Secale cereale*

Plant	Mean	Mean squares		x	No. of nuclei
		Within nuclei	Between nuclei		
1	2·1600	0·2476	0·1824	0·2633	25
2	2·2171	0·3352	0·2110	0·3705	25
3	2·0800	0·2400	0·2033	0·1529	25
4	2·1371	0·3610	0·1486	0·4499	25
5	2·2800	0·3829	0·3271	0·1457	25
6	2·7257	0·6057	0·3324	0·4512	25
7	2·4800	0·4264	0·3319	0·2216	25
8	2·4400	0·4952	0·3681	0·2567	25
9	2·0971	0·4857	0·1038	0·7863	25
10	2·2000	0·3771	0·2662	0·2941	25
11	2·7829	0·5219	0·3943	0·2445	25
12	2·2000	0·4966	0·4975	−0·0018	35
13	2·7771	0·3131	0·2584	0·1947	33
14	2·1388	0·4082	0·2226	0·4547	35
15	2·5714	0·6435	0·3782	0·4123	35
16	2·5184	0·5701	0·2192	0·6155	35
17	2·3633	0·3061	0·1289	0·5789	35
18	2·4082	0·5299	0·3499	0·3397	35
19	2·3102	0·5959	0·1553	0·7394	35
20	2·3036	0·6270	0·3144	0·4986	24
21	2·2286	0·4548	0·1791	0·6062	30
22	2·2229	0·5295	0·4729	0·1069	25

correlation there is a tendency in that direction. A more precise test of this is possible. The mean of the correlation coefficients is $-0{\cdot}1239$ and the variance of the series of coefficients $0{\cdot}011425$. Hence a variance of the mean is $0{\cdot}0011425$ since we have 10 values. We may use the t distribution to test the significance of the deviation of the mean from zero, which is the mean expected if there is no competition and hence no real correlation

$$t = \frac{\bar{r}}{s_{(r)}} = 3{\cdot}664$$

n, the number of degrees of freedom, is 9.

The probability of getting such a large value of t in an uncorrelated population is less than 1%. Hence we must judge the deviation from zero to be significant and that there is good evidence of the occurrence of negative correlations between the frequencies of chiasma formation in the ring and bivalents of various *Oenotheras*. This view is supported by the remaining two cases in which there were two rings and a number

of bivalents. The method of analysis in these cases was by the analysis of variance. In these cases the analyses of variance could be made complete, and are given in Table IV.

In neither case is there a significant difference between the mean squares between nuclei and inherent, although both cases show the latter as the greater. The z test in one case shows only slightly more than a 5% probability of occurrence in an uncorrelated population. While not providing convincing evidence of negatively correlated chiasma frequencies themselves these two cases do tend in the same direction as the other 10 and hence strengthen the conclusions drawn from the previous results.

TABLE IV

	Dependens–^{h}blandina		Serratens–^{h}blandina	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
Between configurations	2	310·4959	2	239·4035
Between nuclei	40	0·2659	37	0·2686
Inherent	80	0·3043	74	0·3945

Oenothera has complete terminalization of its chiasmata and so the metaphase frequencies are not the same as the diplotene ones. On the other hand, if the original diplotene chiasma frequencies are supposed to be uncorrelated one must then suppose that the degree of terminalization in different bivalents is negatively correlated. It is difficult to imagine how this can be as terminalization is complete. The only conclusion that can be reached is that the original diplotene chiasma frequencies were negatively correlated, possibly more strongly than the reduced metaphase frequencies. Hence chiasma formation in *Oenothera* is competitive in different configurations.

Vicia faba (Mather and Lamm, 1935)—These results have been completely described in the earlier publication to which reference is made. There was no competition between the chiasma frequency of the long M chromosome and the totals of the chiasma frequencies of the five short m chromosomes, as tested by the use of the inter-class correlation method. On the other hand, the analysis of variance (incomplete) showed strong indications of the chiasmata of the individual m chromosomes being competitive. The range in size of the m chromosomes is very small and quite inadequate to explain the observed results.

Allium macranthum (Levan, 1933, and unpublished)—There are three length classes of bivalents in this species as described by Levan. The

long class comprises six bivalents, the medium four, and the short four too. Analysis was by the use of the analysis of variance.

The primary analysis of variance for correlation between the totals of the bivalents in the different classes was done completely. The mean square between nuclei is 0·3625 and the inherent mean square 0·3836. There is clearly no evidence of correlation.

The inquiry was pushed a stage further by analysing the results within each length class. This had to be done by incomplete analysis. The shorts showed no sign of correlated chiasma frequencies, and the mediums but little sign. The longs, however, showed a strong negative correlation. The mean square between nuclei is 0·3304 and within nuclei 1·2792. The test of significance gives a result outside the 1% point of probability. This result cannot be attributed to size differences within the long class since it would then demand a size range overlapping the medium class. Hence chiasma formation in the bivalents of the long class is competitive. The medium and short bivalents show no competitive effect.

Allium zebdanense (Levan, 1935, and unpublished)—Dr. Levan informs me that the nine bivalents can be picked out at meiosis with reasonable accuracy in this plant, and so it is possible to make a complete analysis of variance for all the comparisons.

Four sets of data were available, two from counts at diakinesis and two from metaphase. The results are very variable. Taking all the bivalents into one analysis, one set of data shows a positive correlation, two no correlation, and the other a negative correlation. However, for further analysis the bivalents were divided into two groups, the longest five and the shortest four. On repeating the analysis on these groups of bivalents the longs show a positive correlation in two of the four sets of data, no correlation in one, and a suggestion of a negative correlation in the fourth. The shorts on the other hand show a suggestion of a positive correlation in one case, no correlation in a second, and negative correlations in the other two, in one case the negative correlation being very strong. The difference between the two sets of chromosomes is clear. The longs tend to be uncorrelated or positively so, and the shorts tend to be negatively correlated. The significance of the results is, however, difficult to judge on account of their variability. One may conclude, perhaps, that the long bivalents show competition much less often than the short ones.

Tulipa var. " Pink Beauty " (Darlington and Mather, 1932)—This is an autotriploid variety of cultivated tulip and forms from 9 to 12 trivalents at first division of meiosis. The remaining trivalents are always repre-

sented by a bivalent and a univalent in each case. If we consider the potential trivalent as the unit we can analyse the chiasma frequency results in such a triploid in just the same way as in a diploid, where the bivalent is the unit.

The results of the analysis are very striking. The mean square between nuclei is 0·2128 and within nuclei 1·2017. This indicates a very strong negative correlation and hence strong competition—a result of importance in that triploids in general have a lower interference within the trivalent than do diploids within the bivalent.

Secale cereale (*see also* Mather and Lamm, 1935; and Darlington, 1933)—Data are available for 22 plants from the same population although grown under different conditions. Individually many of the plants show negative correlations when the incomplete analysis of variance is used. These are incapable of being explained on the known chromosome size range of 1 to 1·4.

The existence of data from 22 plants allows of a more complete analysis of the situation. Table III gives the mean number of chiasmata per bivalent and the within- and between-nuclei mean squares of each of these plants. Now a question of prime importance is whether the mean square between nuclei is or is not related to the mean square within nuclei, or to the mean. This can be tested by calculating the partial regression coefficients of the former mean square on the within nuclei figures and on the means.

The method of calculating such partial regression coefficients is given by Fisher (1934). Where b_1 is the regression of the mean square between nuclei on the mean square within nuclei, and b_2 its regression on the mean number of chiasmata

$$b_1 = 0{\cdot}2739 \quad \text{and} \quad b_2 = 0{\cdot}1384.$$

The t test of significance as described by Fisher gives

$$t_{b_1} = 1{\cdot}245 \qquad n = 19 \qquad \mathrm{P} = 0{\cdot}3\text{–}0{\cdot}2,$$
$$t_{b_2} = 1{\cdot}005 \qquad n = 19 \qquad \mathrm{P} = 0{\cdot}4\text{–}0{\cdot}3.$$

Neither regression coefficient may be judged to be deviating significantly from 0. This conclusion is strengthened by the fact that the total sum of squares of the between nuclei determinations is 0·2528 whereas that remaining when the portions appertaining to the two regressions are removed is 0·1931. The regression accounts for a very small proportion of the total variance of these mean squares.

The mean squares between nuclei cannot be judged to be correlated with either the mean square within nuclei or the mean numbers of chiasmata.

One direct implication of this result may be stated here, viz., that this removes the basis for supposing that the negative correlation is false and due to size differences since, if that were true, the two mean square series must be correlated. The other implications of these results will be considered in the next section.

The Hypothesis of Competition

The outstanding features of the foregoing observations may be summarized as follows:—

(i) Positively correlated chiasma frequencies seldom occur and may be attributed to mixed material, or environmental effects.

(ii) Negatively correlated chiasma frequencies do occur more often but are not universal. Their occurrence or absence appears to bear no relation to the taxonomic groups of the organisms.

(iii) Some species fail to show negatively correlated chiasma frequencies, others show them in some individuals only and a further group shows them with respect to some bivalents and not others.

(iv) A comparative analysis of 22 rye plants shows that the mean square between nuclei is uncorrelated with either the mean square within nuclei or the mean.

It might be supposed that the negative correlation of chiasma frequencies of different bivalents is but another effect of the system which determines the negative correlation within bivalents, *i.e.*, interference. That this cannot be so is shown by the uncorrelated chiasma frequencies of the bivalents of some organisms which show quite strong interference, *e.g.*, *Culex*, *Crocus*. In general it may be said that interference as shown by Haldane's method is universal in occurrence whereas the negative correlation between bivalents is not. This is confirmed by the rye results since on this hypothesis the between- and within-nuclei mean squares should be correlated, as both interference and competition values are functions of the mean square within nuclei.

The clue to the real situation is provided by the rye results. The variance between nuclei must be determined by a system different than that determining interference. This system clearly tends to stabilize the number of chiasmata forming in the nucleus in spite of the variation in the individual bivalents.

Now Lamm (1936) has shown that, while normal rye often shows negative intra-class correlations, inbred rye with a lower mean chiasma

frequency seldom shows such correlations. The stabilizing influence is more marked when the number of chiasmata is higher. This points to the conclusion that there is an upper limit to the number of chiasmata which can form in any nucleus, the limit varying slightly from nucleus to nucleus. The bivalents or other configurations must compete for these chiasmata. What determines the limit is not known, but there is presumably something, necessary for chiasma formation, present in limited quantity. Further experimental work may elucidate this point. Various conjectures may be made but our general knowledge of the factors conditioning chiasma formation is insufficient to allow of further profitable discussion now.

It is now important to see how far the hypothesis of limited chiasma formation applies to the results obtained from the plants other than the rye.

In the first place, if this hypothesis is correct it would demand that when one chiasma forms in one bivalent it would reduce the chance of chiasma formation elsewhere by one or, more probably, something slightly less than one, when the limit is approached. In *Allium zebdanense*, we may take out the means of chiasma formation in nuclei when various numbers of chiasmata form in any one bivalent. This is done in Table V. It will be seen that where there are signs of negative correlations, as determined by the analysis of variance, there is a drop of about 1·0 chiasma in the remaining bivalents for every chiasma formed in a bivalent which is concerned in the negative correlation. This is clearly of the correct order of magnitude. In the other cases, where the analysis of variance has been used but where it is impossible to distinguish particular bivalents we may use the fact that

$$V(T) = (1 - x)\, V(B),$$

where V (T) is the variance between nuclei and V (B) the variance within nuclei, and x the amount by which the formation of one chiasma reduces the chance of formation of another.

The value of x is given for the various cases in Tables III and V. In rye, where the plants sometimes show competitive chiasma frequencies and sometimes do not, x varies between $-0{\cdot}0018$, and $0{\cdot}7394$. In the other species showing competition x varies from $0{\cdot}5$ to $0{\cdot}8$. These values are again of the correct order of size.

Secondly, one would expect that, in general, the lower the number of chiasmata formed in the nucleus the less the likelihood of the results showing the effects of competition. Hence where either the number of bivalents

TABLE V—*Allium zebdanense*

Source of data	No. of chiasmata	Bivalents I		II		III		IV		V		VI		VII		VIII		IX	
1	1	1	17·00									10	16·90	14	16·79	12	17·08		
	2	4	14·75	6	14·83	12	15·33	16	15·25	19	16·05	10	16·10	6	16·50	8	15·88		
	3	6	15·50	10	15·10	6	16·17	4	15·50	1	14·00								
	4	9	14·78	4	15·50	2	14·50												
2	1													6	20·00	7	19·00		
	2			2	15·50	3	17·33	8	17·75	7	16·86			6	18·17	5	19·40		
	3	6	16·33	7	17·86	7	17·86	2	18·50	5	18·60								
	4	3	17·33	3	18·00	2	17·50	2	19·00										
	5	3	17·33																
3	1											3	16·67	7	16·71	6	16·67		
	2	1	15·00	5	15·40	4	15·50	8	16·00			7	15·86	3	16·33	4	16·00		
	3	7	14·57	5	15·20	6	15·00	2	14·00										
	4	2	15·00																
4	1													4	16·25	5	16·40		
	2	5	15·40	4	15·50	4	15·75	5	15·20	8	15·75			6	17·33	5	17·40		
	3	2	15·50	5	16·00	6	15·83	5	16·20	2	18·00								
	4	3	16·00	1	15·00														

The second column gives the number of chiasmata in the bivalent under consideration. The third–eleventh columns give the mean number of chiasmata in the remaining eight bivalents when the particular bivalent has any given number of chiasmata. In each of these columns the first number is the number of nuclei, on which the mean chiasma frequency is based, and the second number the chiasma frequency.

The portions of the table with double lines are those corresponding to the analyses of variance which indicate negative correlations.

is small or the mean chiasma frequency per bivalent is low, one would not expect to find negatively correlated chiasma frequencies. This is borne out by the observations. The group showing no competition, Table II, has on the whole a lower number of chiasmata per nucleus than those showing competition, Table III. This, in *Crocus* and *Culex*, is coupled with a very small number of bivalents and, in *Crocus Kowolkowii* with 10 bivalents, a low mean number per bivalent. The organisms with competition have a higher number of bivalents or a high chiasma frequency, as in *Vicia faba*. This comparison of the organisms with and without negatively correlated chiasma frequencies is, of course, a very broad one. The results are, however, definitely in favour of the hypothesis of competition.

The question of the relation of interference and competition is less easy to discuss. It would appear from the triploid tulip data that an organism with low interference, *i.e.*, high ratio of the within nuclei variance to the mean, has strong competition. This is rather to be expected on the present hypothesis, as the effects of limitation of the total would be more marked. However, it is not always easy to say whether the variance within nuclei is due to the effects of correlation with other competing bivalents, or whether the competition is an effect of the difference in variances. One thing is clear, viz., that we cannot suppose that all bivalents in a nucleus have precisely the same value as competitors particularly where size differences exist. Where one can pick out two or more classes of bivalents in the nucleus, one would expect to find them affected differentially by the competition. In other words one would expect to find some showing competition and others not; this is so. Wherever size differences exist the size classes show differences in this respect. The precise effect is more difficult to ascertain. In *Allium macranthum* the long bivalents show the correlation, in *Vicia faba* the shorts are the ones with correlated frequencies and so on. One rather instructive case is that of eight chromosome rye (Darlington, 1933).

Now if the negatively correlated chiasma frequencies are due to such a limit to the number of chiasmata in a nucleus, it is clear that, in cases where two or more classes of configuration can be distinguished, if all show negative correlation within themselves they must all have approximately the same between nuclei mean square unless different classes compete for different things—an unsupported supposition. This occurs in *Allium zebdanense* 3 which is the only one of this type available. Where one class shows a correlation and the rest do not, either of two results might be expected. First the between nuclei and within nuclei mean squares for the uncorrelated class might be the same and larger than

the mean square between nuclei for the correlated class. Or secondly the between-nuclei mean squares for all the classes might be the same but the within-nuclei mean squares for the uncorrelated classes less than this figure, and the within-nuclei mean squares for the correlated class greater. *Allium macranthum* may be cited as an example of this type. These two cases clearly indicate that they would result from different situations. In the one case the lack of correlation in some class is due to that class being unaffected by the competition for chiasma. In the other case although there might be competition this would be masked in some classes by the large variation from nucleus to nucleus of the limiting number of chiasmata.

In normal rye the seven bivalents show competitive chiasma formation. In eight chromosome rye the seven normal bivalents show no competition, but the chiasma frequency of the distinguishable short bivalent is correlated with the total of the seven long ones (*see* Mather and Lamm, 1935). This would definitely indicate that the short bivalent is at a disadvantage with respect to the long ones, and furthermore since the short one does form some chiasmata and yet the long ones show no competition among themselves, the introduction of the short bivalent must somehow have raised the limiting number of chiasmata of the nucleus. A study of trisomic types would help to clear up this point.

If there is a large size discrepancy between the different bivalents of an organism the length-chiasma frequency relation is not linear, all bivalents having at least one chiasma (Darlington and Dark, 1932). The short bivalents have a higher mean number of chiasmata per unit length than the others. These bivalents would appear to be forming their chiasmata unaffected by the other bivalents, or else to be better fitted for chiasma formation in a competitive system than the rest. This last postulation does not seem so probable when we consider that in a number of the species dealt with above, *e.g.*, $n = 8$ rye, the shorter chromosomes are less able to form chiasmata in the competitive system. Hence we might expect organisms with the non-linear chiasma frequency length relationship never to show competitive chiasma formation in different bivalents. As far as can be judged this is so. Darlington (1933) says that he found no indication of correlation in data from *Chorthippus elegans*, and the same is true of *Locusta migratoria*, and *Crocus Kowolkowii*, Table II. On the other hand, those organisms which show negative correlations, notably rye, do often show univalent formation, *i.e.*, failure of chiasma formation in some bivalents although there is no great size range among the chromosomes. Thus it would appear that one of the conditions for the regular formation of chiasmata by all the bivalents in a nucleus

irrespective of the size range is the absence of competitive chiasma formation.

Thus the hypothesis of competition for a limited number of chiasmata s well able to explain the apparent inconsistencies shown by the data. It covers the cases with and without negatively correlated chiasma frequencies, whether in the same or different species. It also leads one to expect that such dependent chiasma formation may exist between some bivalents and not between others in the same nucleus, an expectation which is fulfilled by several organisms. This is perhaps one of the strongest points in favour of the hypothesis, as such observations, when first made, seemed to be very inconsistent.

So far no evidence has been obtained for the supposition of more than one essential material being present in a limited quantity. Such evidence would be provided by the occurrence of competition separately within two distinct groups of configurations and no competition between the groups. Such a situation would be most instructive if found.

Summary

The previously existing evidence as to the occurrence of an effect of crossing over and chiasma formation in one bivalent being partially dependent on these processes in other bivalents of the same nucleus was inconsistent. The present work was undertaken in order to accumulate more evidence as to the extent of occurrence of this phenomenon, and to attempt an analysis of the mechanism behind it. Data were obtained from 27 species and hybrids comprising certain insects and flowering plants.

Positive correlation of the chiasma frequencies of bivalents was found in a few cases and ascribed to environmental effects.

Negatively correlated chiasma frequencies occur more often, but are not universal. The presence or absence of a negative correlation appeared to bear no relation to the taxonomic group of the organism. Some species showed no correlation, some had individuals with and others without correlations, and some even had correlations between some bivalents and not between others of the same nuclei. Negatively correlated chiasma frequencies are termed competitive, in the light of a hypothesis developed from these results.

This competition cannot be due to an extension of the well-known interferences, the two being independent in occurrence.

Inbred rye with a low chiasma frequency fails to show competition whereas ordinary rye with a higher chiasma frequency shows it often.

This is taken to indicate that the total number of chiasmata in a nucleus is limited, but by some condition other than that determining interference. This means that, after a certain level, the bivalents must compete with one another in the formation of chiasmata. Hence the lower the number of chiasmata formed the less the likelihood of finding negatively correlated chiasma frequencies. The data show that species with a low number of chiasmata per nucleus, *i.e.*, low number of bivalents or low mean number per bivalent, seldom show competition whereas those with a high number per nucleus, high number of bivalents, or high mean per bivalent, often show it, Tables II and III.

From this hypothesis it is concluded that where bivalents in the same nucleus have very different frequencies of chiasma formation, there may, or even must, exist differences in the competing power of the different bivalents. Hence with bivalents of the same nucleus showing different chiasma frequencies one expects to find some showing negative correlations and others showing independence. This is borne out by what at first appears to be one of the chief inconsistencies of the data.

The precise nature of the limiting material of the nucleus is not discussed as further work is essential for fuller analysis of the situation.

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The Response of the Kidney to an Alkalosis During Salt Deficiency

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One of the functions of the kidney is unquestionably to protect the body against internal fluctuations of hydrogen ion concentration. It is well known that large doses of alkalis or a period of overbreathing lead to an alkaline urine, and the mechanism involved may be twofold. In the first place an increased amount of base may be excreted, and the way in which BH_2PO_4 in the urine gives place to B_2HPO_4 is an example of this. In the second place the reaction of the urine may be changed by acid radicle substitution, *e.g.*, HCO_3 may be excreted instead of Cl. The latter involves no increased excretion of base.

Vomiting leads to a loss of hydrochloric acid so that the urine tends to become alkaline, and persistent vomiting, which invariably causes a fall in the serum chlorides and a rise in the alkali reserve, should theoretically be accompanied by an alkaline urine—and often is so in practice (Dixon, 1924; Hoff, 1932; Glass, 1932; Katsch and Mellinghoff, 1933; Rachmilewitz, 1934; etc.). On the other hand, acid urines have repeatedly been reported even in the presence of proved alkalosis in the tissues. Thus Brown, Eusterman, Hartman, and Rowntree (1923) recorded that tetany and an acid urine might be found to coexist in the same person. Dixon (1924) described a patient with tetany whose plasma bicarbonate was 101 vols %, but who had an acid urine. Gollwitzer-Meier's (1924) patient had tetany, an alkali reserve of 89 vols %, and a urine of p_H 5. Similar cases have been reported by Odin (1928) and Steinitz (1928), and rather less striking ones by Achard (1930) and Meyer (1931, 1932). Several of these authors have commented on the strange anomaly, and Gamble and Ross (1924–25), Gollwitzer-Meier (1924), and Hartmann and Smyth (1926) have all made the further important observation that the urine became alkaline during recovery or after sodium chloride had been given. Few authors have attempted any explanation of their findings and Gollwitzer-Meier's suggestions that the alkali was fixed by the tissues or that the autonomic nervous system was responsible can hardly be accepted as satisfactory. Odin (1928, quoted by Brandberg)

1929), who described a patient with alkalosis, an acid urine, and a blood-urea of 112 mg per 100 cc, regarded the inability of the patient to secrete an alkaline urine as part of the general renal failure. Such patients, however, may show an abnormally high nitrogen excretion so that the renal failure may be much less severe than the blood-urea would indicate.

Hartmann (1929) pointed out the danger of treating persistent vomiting with alkalis under the impression that such treatment was indicated because the urine was strongly acid.

It is well known that persistent vomiting may bring about not only a loss of free hydrochloric acid but also of neutral salt. Loss of the latter may be very pronounced and this must inevitably lead to a sodium deficiency. In any such deficiency so little sodium may be available that it was thought that the kidney, even during an alkalosis, might not be able to excrete enough of this metal to alter the reaction of the urine. The observation that the urine may become alkaline after the administration of sodium chloride supports the view that the acid urines met with pathologically are merely one expression of the sodium deficiency of the patients. At the same time this explanation is incomplete until the behaviour of the potassium ion has been investigated. What fraction, for example, of the increased base excretion produced by an alkalosis is normally due to potassium? If the fraction is appreciably large, why does not this increased excretion of potassium turn the urine alkaline even during sodium deficiency? It was decided therefore to investigate the problem experimentally on normal healthy persons.

Present Investigation

a—Methods and Subjects

Of the two methods by which an alkalosis can be produced without administering alkalis, overbreathing was selected in preference to continuous removal of gastric juice as being more rapid and severe and less disturbing to the course of the main experiments (McCance, 1936). The overbreathing was regulated by an observer, who counted the respirations aloud minute by minute and noted their depth. In this way rate and depth were kept fairly regular on the first occasion on which each person overbreathed, and a standard was set for subsequent overbreathing by the same person. Tetany appeared regularly during the overbreathing. The time required to produce it differed from one person to the next, but was remarkably constant in the same person on each occasion. Moreover, an observation repeatedly made has been that the cramps of sodium

deficiency do not interfere with, or modify in any way, the tetany caused by an alkalosis. These experiments therefore lend no support to the view that tetany has any connexion with sodium metabolism.

As far as possible each person overbreathed at the same time of day, but this time varied from one person to another. Water was taken periodically for 2 hours before and also during the overbreathing. Urine was passed just prior to the commencement of overbreathing and as soon afterwards as possible. Later specimens were also collected. The p_H of the urines was taken by the B.D.H. capillator method as soon as it was passed. Phosphorus in urine was determined by the Bell and Doisy (1920) method, sulphates according to Fiske (1921), and other constituents as described by McCance (1936).

Four human subjects, three of whom were described by McCance (1936), were made salt deficient by diet and sweating. The fourth was a man (D. W.) aged 23. P. M. E., a woman aged 23, overbreathed for 30 minutes on three occasions, once when in normal health and twice when she was salt deficient. Her overbreathing was not very regular or violent, and she was by no means so salt deficient as the other subjects have been. R. B. N., a man aged 24, overbreathed twice for 30 minutes, once when he was normal and once when he was salt deficient. He proved a good subject and he breathed between 70 and 80 times per minute all through both experiments. R. A. M., a man aged 36, overbreathed for 45 minutes on seven occasions, four times when he was salt deficient and three times when he was in normal health. Of the latter, one set of observations was of little use for the present purpose owing to accidental anoxaemia (McCance, 1935), and on one occasion complete observations were not made. The overbreathing was always deep, rapid, and regular. D. W. overbreathed three times, once when normal and twice when sodium deficient. His overbreathing was regular but not quite so deep or so rapid as that of R. A. M. and R. B. N.

b—Results

Table I contains the results of the overbreathing experiments during normal health. It will be noticed that during overbreathing:

(1) The urine always became alkaline, the greatest change of p_H being from 5·1 to 7·6.

(2) The minute volume rose in every case and there was often considerable diuresis. In the after periods the volumes fell again in two cases and rose further in another.

(3) The urea clearances of D. W. and R. B. N. fell very slightly, and R. A. M.'s appreciably. By the second after-period, R. A. M.'s clearance had returned to normal.

(4) There was no change in the rate of excretion of creatinine.

(5) There was a large increase in the rate of excretion of sodium and potassium. The relative rates at which the two metals were excreted varied considerably both before and during overbreathing, so that the percentage of the increase borne by the sodium varied from 38 to 72%, and in one of R. A. M.'s experiments—not reported in Table I—amounted to 93%. There was no change in the rate of excretion of calcium and magnesium and in any case such small amounts of these divalent metals were excreted that changes in their rate of excretion would scarcely have affected the acid base balance.

(6) There was an increase, sometimes slight and sometimes considerable, in the rate of excretion of chlorides. There was no change in the rate of excretion of sulphates or phosphates during overbreathing although

TABLE I—OVERBREATHING IN NORMAL HEALTH

	P. M. E. 30 minutes' overbreathing		R. A. M. 45 minutes' overbreathing			
	Before	During	Before	During	After (1)	After (2)
Length of period, minutes	100	50	60	60	60	60
p_H of urine	acid	alkaline	5·1	7·6	6·4	—
Minute vol of urine	1·15	5·10	1·36	1·98	2·80	7·10
Urea clearance % of normal (van Slyke)	—	—	94	61	73	96
Rate of excretion—						
Urea mg/min	—	—	18·8	15·8	17·3	22·8
Creatinine mg/min	—	—	1·1	1·2	1·3	1·5
Sodium milli eq/hr	5·95	19·9	9·3	14·1	1·5	4·2
Potassium ,,	1·9	10·5	3·6	11·3	4·0	4·2
Calcium ,,	0·16	0·16	0·28	0·26	0·10	0·30
Magnesium ,,	0·44	0·32	0·30	0·18	0·09	0·40
Chloride ,,	10·4	24·9	9·2	10·5	2·3	3·8
Sulphate ,,	0·85	0·60	1·52	1·66	1·20	1·70
Phosphate ,,	1·40	1·60	1·60	1·60	0·20	1·70
Acid base balance as milli eq/hr	A > B 4·2	B > A 3·8	B > A 1·1	B > A 12·1	B > A 2·0	B > A 1·9
Increase in ratio of fixed base/fixed acid	—	8·0	—	11·0	—	—

TABLE I—(continued)

	R. B. N. 30 minutes' overbreathing			D. W. 30 minutes' overbreathing		
	Before	During	After	Before	During	After
Length of period, minutes	129	40	42	35	37	34
p_H of urine	5·6	8·0	7·4	7·0	7·7	7·1
Minute vol of urine	0·72	1·83	1·52	9·5	14·8	11·2
Urea clearance % of normal (van Slyke)	124	113	115	95	79	82
Rate of excretion—						
Urea mg/min	15·0	18·9	20·0	13·3	11·1	11·7
Creatinine mg/min	1·4	1·2	1·4	1·8	1·7	2·0
Sodium milli eq/hr	6·7	13·0	9·0	32·4	62·5	16·5
Potassium ,,	3·6	11·3	5·9	5·2	17·0	6·8
Calcium ,,	—	—	—	1·6	1·8	0·8
Magnesium ,,	—	—	—	0·95	0·70	0·50
Chloride ,,	8·9	12·4	6·1	31·4	42·5	10·0
Sulphate ,,	2·5	3·0	2·3	3·2	3·1	4·1
Phosphate ,,	0·40	0·40	0·14	0·22	0·22	0·17
Acid base balance as milli eq/hr	A > B 1·5	B > A 8·5	B > A 6·3	B > A 5·3	B > A 36·2	B > A 10·3
Increase in ratio of fixed base/fixed acid	—	10·0	—	—	30·9	—

In calculating the rate of excretion of phosphorus in milli equivalents/hour this element has been assumed throughout to be divalent.

there was a sudden drop in the rate of excretion of the latter by R. A. M. and R. B. N. in the first after-period.

(7) The net result of these changes was a considerable increase in the ratio of fixed base/fixed acid in the urine. This was no doubt the direct cause of the change of reaction.

Table II contains the results of overbreathing during salt deficiency. The results of P. M. E. differ very much from the others and will be discussed separately. This subject was by no means so salt-deficient as the others, nor was her alkalosis so violent. Together these facts probably explain why the response of her kidneys to salt deficient overbreathing was not very different from their response to normal overbreathing. Thus there was a polyuria, no change in the urea clearances, and no constant change in the rate of sulphate or phosphate excretion. Very little sodium or chloride was being excreted but much potassium, and overbreathing increased the rates of excretion of both metals. On the second occasion her rate of excretion of sodium was almost as high *during* overbreathing

TABLE II—OVERBREATHING DURING SALT DEFICIENCY

	P. M. E. (1) 30 minutes' overbreathing		P. M. E. (2) 30 minutes' overbreathing	
	Before	During	Before	During
Length of period, minutes ..	60	37	60	40
p_H of urine	neutral	alkaline	acid	alkaline
Minute vol of urine	2·30	8·10	3·50	6·10
Urea clearance % of normal (van Slyke)	76	92	72	62
Rate of excretion—				
Urea mg/min	18·0	21·7	17·0	14·7
Creatinine mg/min	—	—	—	—
Sodium milli eq/hr	0·28	1·16	0·45	4·58
Potassium ,, ...	13·0	22·0	18·0	34·2
Calcium ,, ...	0·06	0·10	0·07	0·12
Magnesium ,, ...	0·10	0·14	0·03	0·14
Chloride ,, ...	0·70	3·00	0·71	3·22
Sulphate ,, ...	1·50	1·46	1·77	1·30
Phosphate ,, ...	0·92	1·04	2·20	1·30
Acid base balance as milli eq/	B > A	B > A	B > A	B > A
hr	10·3	17·9	13·9	33·2
Increase in ratio of fixed base fixed acid	—	7·6	—	19·3

	R. B. N. 30 minutes' overbreathing			D. W. 30 minutes' overbreathing		
	Before	During	After	Before	During	After
Length of period, minutes ..	55	33	37	32	41	67
p_H of urine	5·3	5·0	5·0	5·4	5·4	5·2
Minute vol of urine	0·47	0·15	0·76	4·60	0·95	1·72
Urea clearance % of normal (van Slyke)	46	22	64	62	36	54
Rate of excretion—						
Urea mg/min	14·8	3·3	24·0	37·0	15·2	30·4
Creatinine mg/min	1·56	0·48	2·50	2·60	1·14	1·67
Sodium milli eq/hr	0·02	0·02	0·07	0·76	0·44	0·44
Potassium ,, ...	2·2	0·7	4·0	4·0	2·9	2·6
Calcium ,, ...	—	—	—	0·27	0·05	0·10
Magnesium ,, ...	—	—	—	0·70	0·10	0·10
Chloride ,, ...	trace	trace	trace	0·11	0·02	0·04
Sulphate ,, ...	2·70	—	5·60	2·20	0·94	2·10
Phosphate ,, ...	1·60	0·26	1·40	2·10	0·36	0·96
Acid base balance as milli eq/	—	—	—	B > A	B > A	B > A
hr				1·32	2·17	0·14
Increase in ratio of fixed base/ fixed acid	—	—	—	—	0·85	—

TABLE II—(continued)

	R. A. M. (1) 45 minutes' overbreathing					
	Before	During	After (1)	After (2)	After (3)	After (4)
Length of period, minutes	60	60	30	30	50	40
p_H of urine	6·3	5·6	5·3	6·0	5·8	5·7
Minute vol of urine	1·14	0·39	1·57	2·50	3·60	5·60
Urea clearance % of normal (van Slyke)	74	32	56	55	68	99
Rate of excretion—						
Urea mg/min	14·0	3·5	12·7	13·5	16·5	24·0
Creatinine mg/min	1·10	0·62	1·53	1·26	1·36	1·70
Sodium milli eq/hr	0·22	0·12	0·19	—	—	—
Potassium ,,	2·45	2·0	2·8	—	—	—
Calcium ,,	0·09	0·04	0·08	—	—	—
Magnesium ,,	0·29	0·10	0·25	—	—	—
Chloride ,,	0·09	0·06	trace	—	—	—
Sulphate ,,	1·40	0·80	2·30	—	—	—
Phosphate ,,	2·00	0·70	0·60	—	—	—
Acid base balance as milli eq/hr	A > B 0·4	B > A 0·7	B > A 0·4	—	—	—
Increase in ratio of fixed base/fixed acid	—	1·1	—	—	—	—

	R. A. M. (2) 45 minutes' overbreathing				
	Before	During	After (1)	After (2)	After (3)
Length of period, minutes	50	60	30	30	60
p_H of urine	6·3	6·4	5·4	6·3	6·0
Minute vol of urine	3·80	0·33	1·50	2·24	3·00
Urea clearance % of normal (van Slyke)	80	33	54	49	68
Rate of excretion—					
Urea mg/min	28·0	5·0	16·6	17·3	24·0
Creatinine mg/min	1·26	0·67	2·00	1·24	—
Sodium milli eq/hr	0·14	0·05	0·11	—	—
Potassium ,,	2·4	1·0	3·3	—	—
Calcium ,,	0·34	0·04	0·22	—	—
Magnesium ,,	0·60	0·10	0·30	—	—
Chloride ,,	0·14	0·05	0·04	—	—
Sulphate ,,	2·04	0·60	3·30	—	—
Phosphate ,,	2·00	0·44	0·30	—	—
Acid base balance as milli eq/hr	A > B 0·7	B > A 0·1	B > A 0·3	—	—
Increase in ratio of fixed base/fixed acid	—	0·8	—	—	—

In some cases it will be observed that the urea clearance calculated by van Slyke's formula falls while the rate of excretion rises. This apparent contradiction is due to the use of the square root formula when the minute volume is less than 2 cc/minute. This also applies to Table III.

as it had been *before* overbreathing during normal health. There was on average a greater increase in the ratio of fixed base fixed acid than there had been when she was normal, but 85% of the increase was due to potassium.

The results obtained on the other subjects were quite different from this and may all be taken together. It will be observed that:

(1) The urine never became alkaline and the p_H was actually lowered in two cases.

(2) The minute volume fell profoundly in every case and rose again in the subsequent periods.

(3) There was a large fall in the urea clearances and the rate of excretion of urea with a subsequent rise in both.

(4) The rate of excretion of creatinine fell in all cases during overbreathing, and subsequently recovered. The rates of excretion during overbreathing were only 1/3 to 5/8 of their previous values.

(5) There was actually a decrease in the rate of excretion of sodium and potassium. There was also a fall in the rate of excretion of calcium and magnesium.

(6) There was a diminished rate of excretion of both sulphates and phosphates during overbreathing with subsequent recovery in the case of the former and sometimes also of the latter.

(7) There was little change in the relative rates of excretion of fixed base and fixed acid. The small increase in the ratio of fixed base fixed acid in the urine was due to the drop in the sulphate and phosphate excretion.

Discussion

These results constitute the experimental reproduction under controlled conditions of the scattered clinical observations to which reference has already been made. It is evident that an alkalosis during sodium deficiency, even if severe, may not produce an alkaline urine.

Why does the urine not become alkaline? In the first place it is quite possible that abnormally large amounts of organic acids may be produced during salt deficient overbreathing, and this would tend to raise the rate of excretion of acid bodies. Whether this is so or not, it is certain that there is no increased excretion of fixed base, which is such a characteristic feature of normal overbreathing. Owing to the deficiency of sodium, little of this base is available for excretion at any time, and its rate of excretion can never be raised enough to alter appreciably the p_H of the urine. The kidney prefers to regulate the osmotic pressure of the plasma

at the expense of its p_H. In normal overbreathing the rate of excretion of potassium may be increased 300 to 500%. During salt deficient overbreathing there is no deficiency of potassium and yet its rate of excretion not only fails to rise, but actually falls. Hence, the urine remains acid because there is no increased excretion of fixed base.

Why does the rate of excretion of fixed base fall during salt deficient overbreathing? Probably because of the generalized depression of excretory function which accompanies overbreathing during salt deficiency, and which is illustrated by the fall in the rates of excretion of creatinine, urea, sulphates, phosphates, and possibly water.

What causes this generalized depression of excretory activity? The experiments so far described do not make this clear, for the results might equally well be attributed either to the mere muscular exertion of overbreathing or to the alkalosis. As regards the former, salt deficient subjects have a blood volume which is certainly subnormal; and it was thought therefore that some reduction in the *renal* circulation might have to accompany the exertion of overbreathing to allow the necessary increase in the *muscular* circulation to take place.

In order to decide between these alternatives, a control experiment was performed on two of the subjects. D. W. and R. A. M. overbreathed while they were salt deficient, using a CO_2-air mixture to prevent any alkalosis. It was found quite easy to do this, and the CO_2 supply was controlled voluntarily by the overbreather. Overbreathing of this sort was found by both subjects to be very monotonous and they required periodic stimulation. The rates and depth of breathing, however, were fully maintained. There was no suggestion of an alkalosis. The results are shown in Table III. It will be observed that:

(1) There was, as expected, no significant change in the p_H of the urine.

(2) There was an inconstant variation in the minute volume. R. A. M.'s minute volumes tended to fall on both occasions in the first after-period.

(3) There were very slight falls in the urea clearances and rates of urea excretion.

(4) There was no change in the rate of excretion of creatinine, sulphates, or phosphates.

(5) There was no important change in the rates of excretion of the fixed bases.

Hence, no striking renal changes took place during overbreathing when alkalosis was prevented by the administration of CO_2. The generalized depression of renal excretion which occurred on other occasions (Table II) must therefore be attributed to the alkalosis and not to the overbreathing *per se*.

TABLE III—OVERBREATHING WITH CO_2 DURING SALT DEFICIENCY

	D. W. 30 minutes' overbreathing			
	Before	During	After (1)	After (2)
Length of period, minutes ..	33	37	35	30
p_H of urine	5·6	5·6	5·5	5·4
Minute vol of urine	3·10	2·10	2·15	2·23
Urea clearance % of normal (van Slyke)	53	43	43	42
Rate of excretion—				
Urea mg/min	27·6	23·0	22·5	22·2
Creatinine mg/min	2·40	2·00	1·93	2·04
Sodium milli eq/hr	0·62	0·56	0·40	0·50
Potassium ,, ...	2·40	2·55	2·50	2·70
Calcium ,, ...	1·00	0·93	0·80	1·02
Magnesium ,, ...	0·45	0·25	0·29	0·40
Chloride ,, ...	0·05	0·04	0·03	0·04
Sulphate ,, ...	1·30	1·10	1·20	1·50
Phosphate ,, ...	0·72	0·56	0·96	1·80
Acid base balance as milli eq/hr	B > A 2·4	B > A 2·6		

	R. A. M. (1) 45 minutes' overbreathing				R. A. M. (2) 45 minutes' overbreathing			
	Before	During	After (1)	After (2)	Before	During	After (1)	After (2)
Length of period, minutes ..	45	49	47	51	52	49	44	47
p_H of urine	6·2	6·1	6·0	5·8	6·2	6·0	5·7	5·6
Minute vol of urine	1·40	2·40	0·78	1·93	0·96	1·00	0·71	1·06
Urea clearance % of normal (van Slyke)	75	65	74	51	68	52	50	38
Rate of excretion—								
Urea mg/min	21·7	21·0	15·6	17·2	16·3	12·5	10·2	9·4
Creatinine mg/min	1·63	1·65	1·40	1·62	1·70	1·61	1·20	1·45
Sodium milli eq/hr	0·13	0·23	0·08	0·18	0·13	0·26	0·21	0·24
Potassium ,, ...	1·50	1·30	0·90	1·70	1·20	1·25	1·20	1·35
Calcium ,, ...	0·12	0·33	0·15	0·12	0·15	0·39	0·10	0·54
Magnesium ,, ...	0·25	0·65	0·35	—	0·35	0·80	0·25	0·15
Chloride ,, ...	0·12	0·14	—	0·14	0·07	0·14	0·10	0·04
Sulphate ,, ...	1·10	1·20	1·00	2·00	1·20	1·30	0·94	0·94
Phosphate ,, ...	0·48	1·20	1·30	1·90	0·84	1·50	1·20	1·40
Acid base balance as milli eq/hr.....	B > A 0·30	A > B 0·03			A > B 0·28	A > B 0·24		

It remains to consider why the response of the salt deficient kidney to an alkalosis is so different from that of a normal one. This may be merely a matter of degree, for the way in which R. A. M.'s urea clearance fell during normal overbreathing suggests that even short periods of alkalosis may depress the activity of some normal kidneys. It is known that kidneys, supposedly normal, may be functionally disorganized by the continuous administration of alkalis (Berger and Binger, 1935).

Patients with signs of nephritis do not excrete large doses of alkalis in the normal way (Berger and Binger, 1935; Oakley, 1935), and prolonged administration may greatly reduce their functional renal capacity. From evidence to be presented later it is known that the salt deficient kidney is functionally below par, but there is no nephritis in the clinical sense, and there are extra-renal causes to account for the subnormal function. Why then should it be so sensitive to an alkalosis?

The whole subject is very obscure (Cooke, 1932; Ryle, 1935; Oakley, 1935), but it is perhaps worth noting that, apart from the alkalosis, the only chemical finding common to both salt deficiency and an uncompensated alkalosis is the low plasma chloride. It might be worth trying to prevent uncompensated alkalosis developing in patients undergoing alkaline treatment by giving them sodium chloride. Whatever the reason, the fact remains that the kidneys of a salt deficient person function very badly in the presence of an alkalosis.

The present experiments do not settle whether the alkalosis produced the fall in renal excretion by a direct action on the kidney or by some indirect, possibly cardiovascular, means. The simultaneous fall in the rates of excretion of creatinine and sulphates and in the urea clearance suggests decreased glomerular filtration, but there is no evidence as to how this may have been brought about. The whole question of alkalosis during salt deficiency is under investigation.

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Summary

Overbreathing by normal persons produced an alkaline urine, an increased excretion of both sodium and potassium, and a diuresis.

The function of the normal kidney was not found to be generally depressed by a short term alkalosis, but the urea clearance of one subject fell significantly.

Overbreathing by salt deficient persons produced no change in the p_H of the urine, no increased excretion of potassium or sodium, an oliguria, and a generalized lowering of functional renal activity as shown by falls in the rates of excretion of creatinine, urea, sulphates, and phosphates.

When alkalosis was prevented by the administration of CO_2 during salt deficient overbreathing, no generalized depression of excretory activity was observed, which indicated that the latter was directly due to alkalosis and not to overbreathing.

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The Abnormal Structure Induced in Nodules on Lucerne (*Medicago sativa* L.) by the Supply of Sodium Nitrate to the Host Plant

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[Plates 9–11]

1—Introduction

It was shown in a recent paper (Thornton and Nicol, 1936) that the application of sodium nitrate to inoculated lucerne grown in sand, produced two effects upon the development of nodules. Firstly, the number of nodules was reduced and, secondly, their mean size was diminished. Both these effects increased with the nitrate dose, but, with weak doses of nitrate, it was the reduction in mean nodule size that principally affected the total mass of bacterial tissue carried by the host plant. Nevertheless, the action of nitrates in reducing the number of nodules, that is, their influence upon root infection, has occupied the attention of many workers, whereas but few have studied the growth of nodules on roots supplied with nitrates.

The action of nitrate in diminishing root-hair infection by the nodule organism was recently investigated by one of us (Thornton, 1936). Infection is preceded by an increased and irregular growth of the root-hairs which is induced by secretions of the bacteria. Without this irregular growth the root-hairs remain uninfected. Nitrate inhibits this action of the bacterial secretions in stimulating irregular root-hair growth, and so checks infection. The action of nitrate upon legume root-hairs is thus superficially analogous to its action upon the already formed nodule, where it inhibits or checks the growth, which is normally stimulated by the presence of the contained bacteria. Only by a close study of the detailed effects of nitrate upon nodule growth could the significance of this analogy be disclosed. The action of nitrate in reducing the irregular growth of root-hairs exposed to the sterile secretions of nodule bacteria, can to some extent be counteracted by the simultaneous supply of dextrose to the roots (Thornton, 1936). This suggests that the

inhibitory action of the nitrate upon root-hair growth is an indirect one, due to the building up of protein within the plant resulting in a shortage of carbohydrate supply to the root-hairs. One might thus expect, by analogy, that the reduction of nodule growth in a nitrate-fed plant could also be explained as being due to carbohydrate deficiency. Fred and Wilson (1934) indeed found that the size of individual nodules on soybeans was reduced by sodium nitrate manuring, but that this effect could largely be overcome by enriching the carbon dioxide supply to the leaves. This hypothesis would be supported if the structure of nodules on nitrate-manured plants showed evidences of carbohydrate shortage. A somewhat different explanation of the nitrate effect was put forward by Giöbel (1926) who supposed that the concentration of nitrate in the tissues of the host plant checked the removal of the products of nitrogen fixation, which thus accumulated in the nodule until they become toxic to the bacteria. On this hypothesis, nodules on plants given nitrate should perhaps show evidence of the accumulation of nitrogenous compounds, such as protein, in the nodule cells. It seemed, therefore, that a comparison of the detailed structure of nodules on plants grown with and without nitrate might supply facts, by which the above hypotheses could be tested, or which would suggest some other explanation of the inhibitory action of nitrate.

2—Technique

The observations described below were made on material obtained by means of the following general method. Lucerne seedlings were grown in an agar medium, referred to below as "seedling agar", having the following composition:—

K_2HPO_4	0·5 gm
$MgSO_4 . 7H_2O$	0·2 ,,
NaCl	0·1 ,,
$Ca_3(PO_4)_2$	2·0 ,,
$FePO_4$	1·0 ,,
$FeCl_3$	0·01 ,,
Agar	10·0 ,,
Water	1000 ml

30 cc of this medium were placed in each of a number of wide test tubes (3 cm diameter) which were plugged with cotton wool and sterilized in an autoclave. Lucerne seeds were sterilized externally by immersion for 3 minutes in absolute alcohol and then for 3 minutes in 0·2% $HgCl_2$, and the antiseptic was washed off with sterile water. About four seeds were

transferred aseptically to each tube of agar medium and inoculated with an active culture of the lucerne nodule organism. When some four or five nodules had appeared on each plant, the latter were transferred aseptically to fresh tubes of sterile medium, melted and kept at 42° C during the transplanting. These tubes contained " seedling agar ", either without addition, or with the additions shown in Table I. Three batches of such transplanted cultures of lucerne were grown in 1932 and 1933, using, throughout, the general technique described above, but with some variation in the composition of the media into which the seedlings were transplanted and in the date and period of growth. These details are shown in Table I.

TABLE I—PLAN OF THE EXPERIMENTAL CULTURES FROM WHICH THE MATERIAL HERE DESCRIBED WAS OBTAINED

Experiment	Date of sowing in medium without nitrate	Date of transplanting	Medium into which seedlings were transplanted: seedling agar plus	Number of replicate tubes	Dates of fixing nodules	Number of nodules sectioned
1932	June 24	July 13	No nitrate	6	Aug. 22	18
			0·1% sodium nitrate	6	Sept. 9	10
			0·2% sodium nitrate	6	Sept. 21	6
1933 I	June 12	July 6	No nitrate	—	July 21	—
			0·05% sodium nitrate	5	Aug. 4	8
			—	5	Aug. 28	8
1933 II	June 25	Aug. 29	No nitrate	6	Sept. 29	9
			0·05% sodium nitrate	6	Oct. 20	8

The cultures both before and after transplanting, were kept in a glasshouse, the lower half of each tube being kept covered with dark paper to protect the roots from light. After varying periods of growth (Table I), the nodules were fixed in Bouin's fixative (Bouin, 1897) and microtome sections were prepared and examined. Most of the material was stained with iron haematoxylin and counterstained with orange G or lichtgrün. Ruthenium red was sometimes employed as a counterstain when studying cell-walls. Some of the material was stained with safranin and lichtgrün, a combination which was found to be particularly useful in showing up suberized cell-walls. Certain microchemical tests were also made on the nodule tissue. These are described in the appropriate places.

3—The Effect of Nitrate Upon the Growth and Structure of the Nodule

It was found that the presence of nitrate in the agar arrested or checked the growth of nodules on the transplanted seedlings. In the 1932 experiment, measurements of nodule length, taken 5 weeks after transplanting, showed no significant differences as between plants grown in 0·1 and 0·2% nitrate, but nodules from these sets were significantly smaller than those on the control plants. Fig. 1 shows the frequency in length of 97 nodules

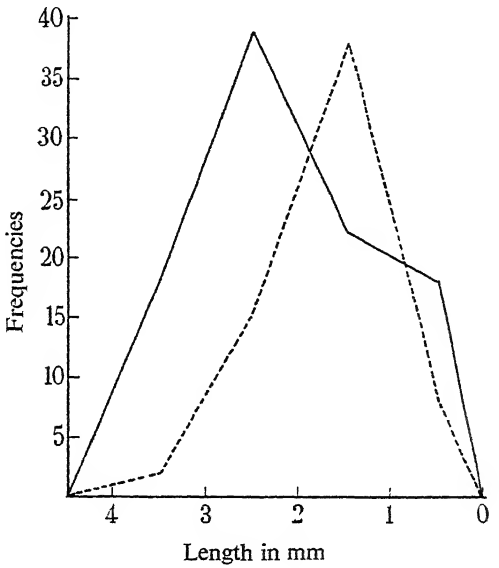

Fig. 1—Frequencies of nodule lengths on lucerne seedlings transplanted and grown for 5 weeks in agar with and without sodium nitrate. —— no nitrate; - - - + sodium nitrate.

from control and 63 nodules from nitrate-fed plants. The former shows a significant excess of nodules longer than 2 mm. The greater number of nodules less than 1 mm on the control plants was due to the appearance of new young nodules, which was inhibited in the nitrate sets. These measurements provide evidence that nitrate checks nodule growth in agar just as it was found to do in sand culture.

The modifications in an organ that are induced by abnormal nutrition commonly vary in individual specimens. Conclusions therefore, should always be based on the examination of a considerable mass of material. This variability, and the consequent need for caution, are especially great in nodules on legumes supplied with nitrate, owing to the difficulty

of standardizing the concentration of nitrate to which individual nodules have been exposed and the length of time during which they have been exposed to it. When plants are grown in agar to which a dose of nitrate has been added, the concentration of this nitrate is continually decreased owing to assimilation by the plant. Towards the end of the experiment, therefore, the nodules will have been exposed to a nitrate concentration far smaller than at its commencement. The method of transplanting was here employed in order to ensure that, in the nitrate sets, the nodules to be examined should all have been exposed at the time of transplanting to nitrate of the desired concentration. With this method, however, the nodules had grown for variable periods in agar without nitrate previous to transplanting, and were of somewhat different ages when first exposed to the effects of nitrate. Thus, on a single plant, nodules were exposed to nitrate during different stages of their growth-period, and to different concentrations of nitrate at any given stage of growth. It was to be expected, therefore, that very variable degrees of abnormality would be found in nodules thus exposed to the influence of nitrate. Evidence that any observed abnormality was the result of the nitrate nutrition must therefore depend on the relative frequency with which this abnormality was found in nitrate-fed and in normal plants. Such numerical data are given in Table II for each of the principal abnormalities described.

There are certain differences amongst publications dealing with nodule anatomy, as to the terms applied to the various parts. Fig. 2 illustrates the meaning of the terms that are used below.

The principal regions of the nodule which showed abnormalities, in the material from nitrate-fed plants, were the distal cap, the lateral endodermis, and the bacterial tissue, figs. 4 and 5, Plate 9.

A—*The Distal Cap*

The healthy lucerne nodule bears at its distal end a cap of meristem cells, by whose continued division the nodule grows in length, fig. 2. The lateral endodermis, vascular strands and other tissues forming the side coating of the nodule are differentiated from the edges of the distal meristem cap. The inner layers in the centre of this cap become successively infected by the bacteria, soon after which they cease to divide, grow in size and eventually become part of the bacterial tissue. Thus the cap remains approximately the same thickness during the healthy growth of the nodule, though, in senescent nodules, the decay of the bacterial tissue (*see* § 3C), eventually affects the cap, whose cells

TABLE II

Experiment	Treatment	Distal cap: Cell-walls: Thin	Distal cap: Cell-walls: With irregular thickening	Distal cap: Nuclei: Normal	Distal cap: Nuclei: Shrunken	Lateral endodermis: Cell-walls: Normal	Lateral endodermis: Cell-walls: Much suberized	Bacterial tissue: Necrotic decay: Absent except near base	Bacterial tissue: Necrotic decay: Extensive	Bacterial tissue: Starch: Present	Bacterial tissue: Starch: Absent	Bacteria: Normal	Bacteria: Cocci predominant	Total number of nodules examined
1932	No nitrate	17	0	14	3	17	1	13	5	9	9	14	4	18*
	+ 0·1% sodium nitrate	8	2	4	6	1	9	5	5	3	7	5	5	10
	+ 0·2% sodium nitrate	4	1	1	4	2	4	2	4	0	6	0	6	6*
1933 I	No nitrate	7	0	7	0	7	1	6	2	4	4	7	1	8*
	+ 0·05% sodium nitrate	0	8	0	8	0	8	1	7	0	8	0	8	8
1933 II	No nitrate	9	0	9	0	5	4	7	2	6	3	9	0	9
	+ 0·05% sodium nitrate	2	6	2	6	0	8	2	6	0	8	1	7	8
Totals from the three experiments	No nitrate	33	0	30	3	29	6	26	9	19	16	30	5	35
	+ nitrate	14	17	7	24	3	29	10	22	3	29	6	26	32

* In each of the three sets indicated there was one nodule whose distal cap was not sectioned.

cease to divide, while their nuclei become shrunken and abnormal. In the material here described, the cells of the healthy meristem cap in control nodules had the normal thin walls. These cell-walls were strongly stained with ruthenium red. They contained a substance having the characteristics of a hemi-cellulose which masked the usual colour reaction for cellulose. Its properties have been investigated by McCoy (1932). After its removal by 24 hours' treatment with eau de Javelle at 25° C, the cell-walls gave typical reactions for cellulose with zinc-chlor-iodide and with I–KI in the presence of H_2SO_4. The cell-contents were usually vacuolated but there was a thick layer of cytoplasm along the cell-walls and round the nuclei. These latter showed some mitotic division; the

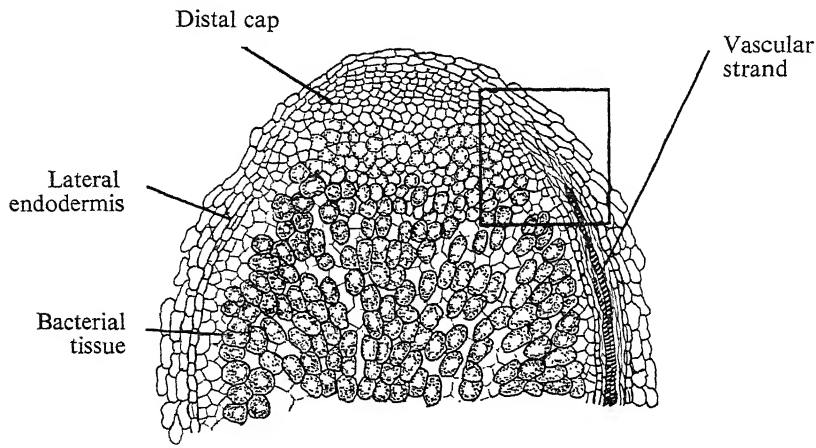

FIG. 2—Distal half of a lucerne nodule, longitudinal section, showing the names here given to the various parts. The square indicates the region that is covered by the drawings shown on Plate 11 (Figs. 12 and 13).

resting nuclei were large, spherical, and generally contained well-developed nucleoli, fig. 5, Plate 9.

In nodules from plants supplied with nitrate, a striking abnormality was often seen in the cell-walls of the distal cap. These walls were greatly thickened and even enlarged to form concretion-like lumps projecting into the cells, fig. 7, Plate 9. These lumps and also the rest of the thickened cell-walls, coloured strongly with ruthenium red and gave the same cellulose reactions after treatment with eau de Javelle as were given by distal cap cell-walls in control nodules. Tests for starch, suberin, and lignin gave negative results. The cell-wall thickenings were thus apparently composed of the same materials as are found in normal cell-walls of this tissue. In the majority of nodules from nitrate-treated plants, the cell contents of the distal cap showed degenerative changes. The cytoplasm

was much reduced and the nuclei greatly shrunken and often irregular in shape. (Compare fig. 12 with fig. 13, Plate 11.) Table II shows, for each treatment, the number of nodules that showed the shrunken nuclei and the irregularly thickened cell-walls in the distal cap tissue.

B—*The Lateral Endodermis*

The mass of infected cells forming the core of the lucerne nodule is bounded laterally by a layer of secondary endodermis. In nodules from control plants, the cells of this layer were usually thin walled, with suberization confined to the Casparian strips. This normal thin-walled endodermis is shown in fig. 8, Plate 10, which photograph does not, however, show the Casparian strips, as the section was stained with iron haematoxylin and orange G. Only in older nodules on the control plants did the suberization tend to involve the whole endodermal cell-wall, this change commencing near the base of the nodule and later extending distally.

In nodules on plants supplied with nitrate, the general thickening of the endodermal cell-walls was usually very pronounced even in quite small nodules, and extended up to the distal cap. It usually involved additional cells, internal to the endodermis itself. Such thickened cell-walls are shown in fig. 9, Plate 10. They could be stained with ruthenium red and gave the same colour reaction for cellulose after treatment with eau de Javelle, as did the distal cap cell-walls. In addition, however, they took on a strong pink colour on treatment for 24 hours with equal parts of an alcoholic solution of scharlach R (sudan IV) and glycerine (Rawlins, 1933). This reaction did not take place if the tissue had been treated previously with alcoholic KOH for 24 hours to remove fatty substance. Tests for lignin by Maule's potassium permanganate method (as given by Rawlins, 1933) and by Peyer's cobalt thiocyanate method (1929) gave negative results whether applied before or after treatment of the material with KOH. The abnormal thickening of the endodermal cell-walls thus appears to have been due to the excessive deposition of suberin, which is a normal component of these walls, although usually confined to the Casparian strips. Here, as in the distal cap, abnormal thickening was due to a local excess of materials that are normal to the tissue.

Table II shows, for each treatment, the number of nodules that showed marked cell-wall thickening in the lateral endodermis.

Closely related to the lateral endodermis in the nodule are the vascular strands, which extend up the sides, between this layer and the bacterial

tissue. Each vascular strand is itself surrounded by a sheath of endodermis, save at its distal extremity, where, in normal nodules, its tissues pass into the meristem of the distal cap.

In nodules on nitrate-treated plants this sheath of endodermis surrounding the vascular strands usually showed abnormally thickened cell-walls extending as far as the distal extremity where they merged with the thickened walls of the distal cap cells. Thus the vessels were sealed off from the rest of the nodule by cell-wall thickenings. This must have affected the supply of food materials to the central tissues of the nodule. The drawings shown on figs. 12 and 13, Plate 11, show the distal extremities of vascular strands and the tissues surrounding them in nodules from control and nitrate-treated plants respectively. The drawings were made from sections longitudinal to the nodules, and the region covered by them is indicated in fig. 2 by the small square.

C—*Bacterial Tissue*

This term is here applied to the mass of cells in the centre of the nodule, most of which cells have their cytoplasm filled with bacteria, fig. 2.

In actively growing nodules on the control plants, the infected cells of the fully developed bacterial tissue usually showed a thick layer of cytoplasm surrounding a central vacuole, this cytoplasm being completely filled with a dense mass of bacteria, fig. 10, Plate 10. Starch grains frequently occurred as a layer lining the cell-wall. In old nodules, the bacterial tissue often showed symptoms of necrotic decay and parasitic attack by the bacteria, especially towards the base of the nodule. This phenomenon is normal to old lucerne nodules and has been discussed elsewhere (Thornton, 1930).

In nodules on plants supplied with nitrate, the infected cells of the bacterial tissue showed a notably thinner layer of cytoplasm and a consequently enlarged central vacuole. Starch was usually absent and the bacteria were fewer in number and so less densely crowded together, fig. 11, Plate 10. Decay of the whole or greater part of the central tissue occurred in many nodules and was more frequent and more extensive than in nodules from control plants, Table II.

The nitrate treatment was also associated with differences in the morphology of the bacteria in the nodules. During the growth of the nodule, the bacterial tissue is continually extended by the infection of cells internal to the distal cap. There is therefore a gradation in age from newly infected cells under the cap to the oldest portion of the bacterial tissue near the base of the nodule. In control nodules the young bacterial

tissue contained bacteria principally in the form of slender rods rather difficult to stain. The infected cells in the centre of the nodule were filled with bacteria most of which had the form of thick rods showing the stainable material sharply segregated into bands and granules.

In nodules on plants given nitrate, the coccus stage of the nodule organism was abundant in the young distal region of the infected tissue, where, in many cells, the organism occurred almost exclusively in this stage. Towards the centre of the bacterial tissue in these nodules the

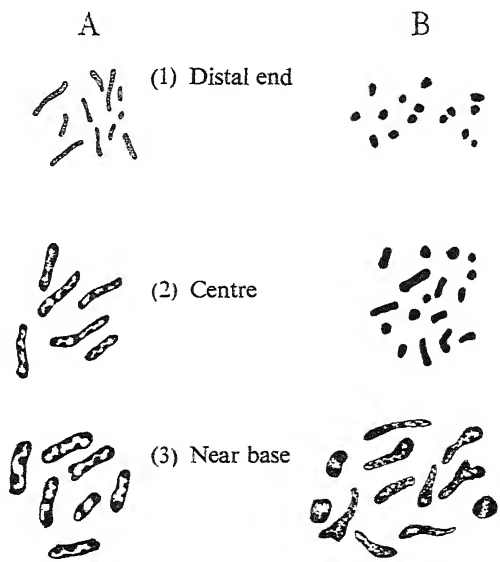

FIG. 3—Bacteria (A) from a normal control nodule and (B) from a nodule from a nitrate-fed plant, showing typical forms found in (1) young infected cells below the distal cap, (2) the centre of the bacterial tissue, and (3) older cells from near the nodule base.

infected cells contained a mixture of cocci and short rods, while in older tissue the cells contained unevenly-staining bacteria somewhat more irregular in shape than were those from a similar position in control nodules.

The prevalence of the coccus stage of the life-cycle in the young bacterial tissue is suggestive of deficient food supply. Bewley and Hutchinson (1919), who called this non-motile coccus the " pre-swarmer " stage, concluded that its appearance in soil was due to a deficiency of food substances.

Typical bacterial cells from corresponding levels in control nodules and from nodules on plants supplied with nitrate are shown in fig. 3.

4—Discussion

The abnormal nodule structures associated with nitrate-nutrition of the host plant fall into two groups. There is (*a*) a remarkable thickening of the cell-walls of the distal cap and a thickening, due to suberization, of the cell-walls of the lateral endodermis. As a result, the infected tissue of the centre of the nodule becomes enclosed and the vascular strands blocked by layers of cells with thickened or suberized walls. There are also (*b*) changes in the nodule tissue suggestive of starvation. Such are the cessation of cell division and shrinking of the nuclei in the distal cap, the reduced cytoplasm and scarcity of starch in the bacterial tissue, the smaller numbers of bacteria and the prevalence of the coccus condition amongst them, and finally the increased tendency to necrotic decay of the nodule tissue accompanied by parasitic attack upon the host-cells by the bacteria, this change to parasitism having been found in previous work (Brenchley and Thornton, 1925; and Thornton, 1930) to be associated with conditions leading to carbohydrate deficiency.

There is no visible evidence of an accumulation within the nodule of the products of nitrogen fixation, such as would accord with the suggestion of Giöbel (1926) that the concentration of nitrates in the plant prevented the removal of these products from the nodule. Rather does the whole appearance suggest a starvation of the internal nodule tissue associated with its enclosure by a layer of suberized and otherwise thickened cell-walls.

When a legume is supplied with adequate nitrogen in an available combined form, it would seem that the growth of nodules upon its roots must be disadvantageous to it, since they consume carbohydrate to no purpose useful to the host plant. In such plants, the enclosure of the infected nodule tissue by a layer of thickened cell-walls could thus be given the same teleological explanation that applies to the analogous isolation of a wound lesion. The present data, however, scarcely enable one to give a satisfactory physiological explanation of the formation of this enclosing layer of thickened cell-walls.

The hypothesis that the effect of nitrate in checking nodule growth is due to a reduction in the supply of carbohydrate to the nodule, is difficult to fit to the present observations since it seems unlikely that such a reduction could result in an excessive deposition of cell-wall material. The nodules on nitrate-treated plants seem to suffer from the misuse of their carbohydrate supply. The interpretation of the observations would seem to depend upon whether the cessation of cell division in the distal cap be regarded as the result or the cause of the deposition of thickened

cell-walls therein. If the first action of the nitrate is to arrest cell division before it stops cell-wall deposition, this might explain the concentration of cell-wall material in the lateral endodermis along which the vascular strands run, and in the distal cap at which they terminate. A satisfactory explanation of the abnormalities found in nodules on plants supplied with nitrate must await a more complete understanding of the causes leading to the formation and maintenance of the distal meristem cap in normal nodules.

The authors gratefully acknowledge assistance received from Mr. J. A. P. Evans and from Dr. Hugh Nicol.

Summary

Lucerne seedlings carrying very young nodules were transplanted into and grown in an agar medium (1) with no nitrate, and (2) containing concentrations of sodium nitrate ranging from 0·05 to 0·2%. The presence of nitrate greatly reduced the growth of the nodules.

Microtome sections of the nodules revealed the following abnormalities which were associated with the supply of nitrate to the host plant:

(1) The cell-walls of the distal cap were very much thickened, the cell-wall material often projecting into the cells in the form of concretion-like lumps. These thickened walls and the lumps gave the same microchemical reactions as did the thin cell-walls of the normal tissue. The cell-contents were usually reduced and the nuclei greatly shrunken.

(2) The endodermis that surrounds the central tissue of the nodule and also that which ensheathes the vascular strands were abnormally thickened by a deposition throughout the cell-walls of material giving the suberin reaction with scharlach R.

(3) There was an increased tendency towards necrotic decay of the central bacterial tissue and, where this did not occur, the cell-contents were usually much reduced.

(4) In the younger portions of the bacterial tissue, the bacteria occur principally in the coccus stage of their life-cycle, a stage usually associated with food deficiency

Description of Plates

Plate 9

Fig. 4—Longitudinal section of a nodule from a control plant given no nitrate. Showing distal cap cells (*d.c.*), lateral endodermis (*l.e.*), bacterial tissue (*b.t.*). × 86.

Fig. 5—Similar section of a nodule from a plant supplied with nitrate, showing distal cap (*d.c.*), and lateral endodermis (*l.e.*) with thickened cell-walls, and bacterial tissue (*b.t.*) with reduced cell contents. × 86.

FIG. 6—Distal cap tissue from a normal control nodule, showing thin cell-walls and large nuclei. × 800.

FIG. 7—Distal cap tissue of nodule from plant supplied with nitrate, showing irregular cell-wall thickenings (*c.w.*), reduced cytoplasm and shrunken nuclei (*n.*). × 800.

PLATE 10

FIG. 8—Part of a longitudinal section of a normal control nodule showing the bacterial tissue (*b.t.*), the thin-walled lateral endodermis (*l.e.*), and the nodule cortex (*c.*). × 352.

FIG. 9—Similar view from a nodule from a nitrate-fed plant showing lateral endodermis (*l.e.*) and adjoining cells, with suberized cell-walls. × 352.

FIG. 10—Bacterial tissue cells from normal control nodule, showing abundant cytoplasm, relatively small central vacuole (*c.v.*), and starch grains (*st.*). × 800.

FIG. 11—Bacterial tissue cells from a nodule from a nitrate-fed plant, showing reduced cytoplasm with consequently enlarged vacuole (*c.v.*) and absence of starch. × 800.

PLATE 11

FIG. 12—Control normal nodule. Drawing of the end of a vascular strand (*v.s.*) passing into the thin-walled meristem cells of the distal cap (*d.c.*) and separated by thin-walled tissue from the nodule cortex (*c.*) and bacterial tissue (*b.t.*).

FIG. 13—Nodule from a nitrate-fed plant. Drawing of the same region as is shown in fig. 12. The vascular strand is ensheathed in endodermis with thickened cell-walls (*l.e.*) and its extremity is sealed by the irregularly thickened cell-walls of the distal cap (*d.c.*).

NOTE—The material from nitrate-treated plants from which the figures were taken, was derived from plants from the 1933 experiments, which were transplanted into agar medium containing 0·05% sodium nitrate.

The drawings were made by H. G. Thornton.

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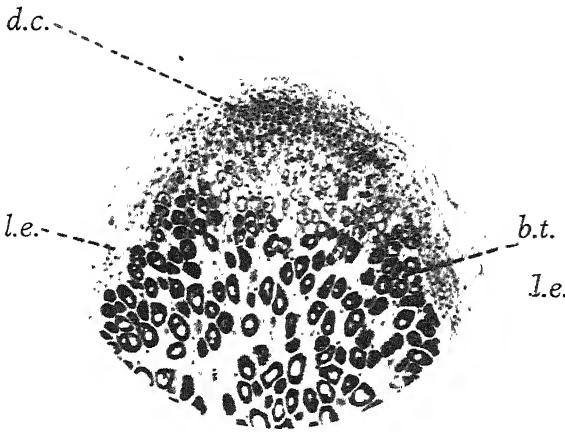

Fig. 4

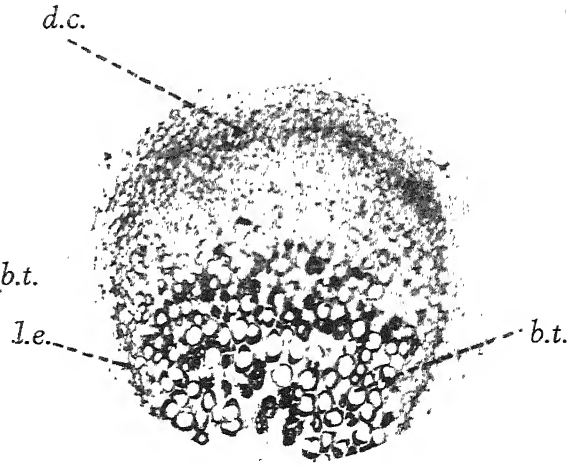

Fig. 5

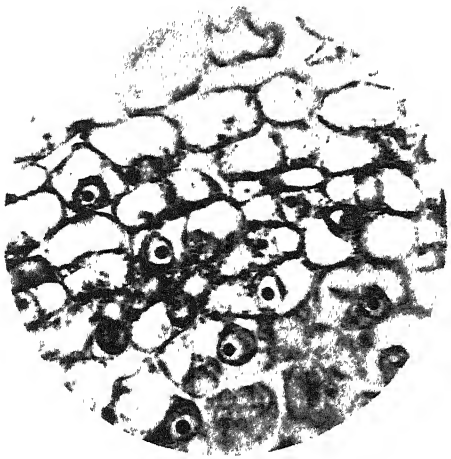

Fig. 6

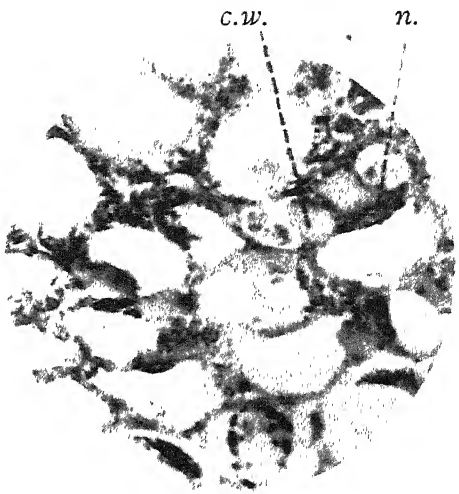

Fig. 7

Fig. 8

Fig. 9

Fig. 10

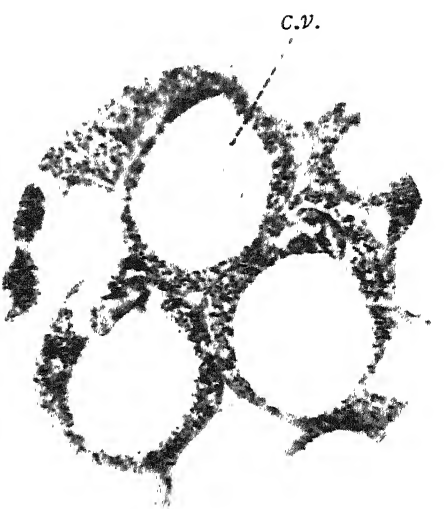

Fig. 11

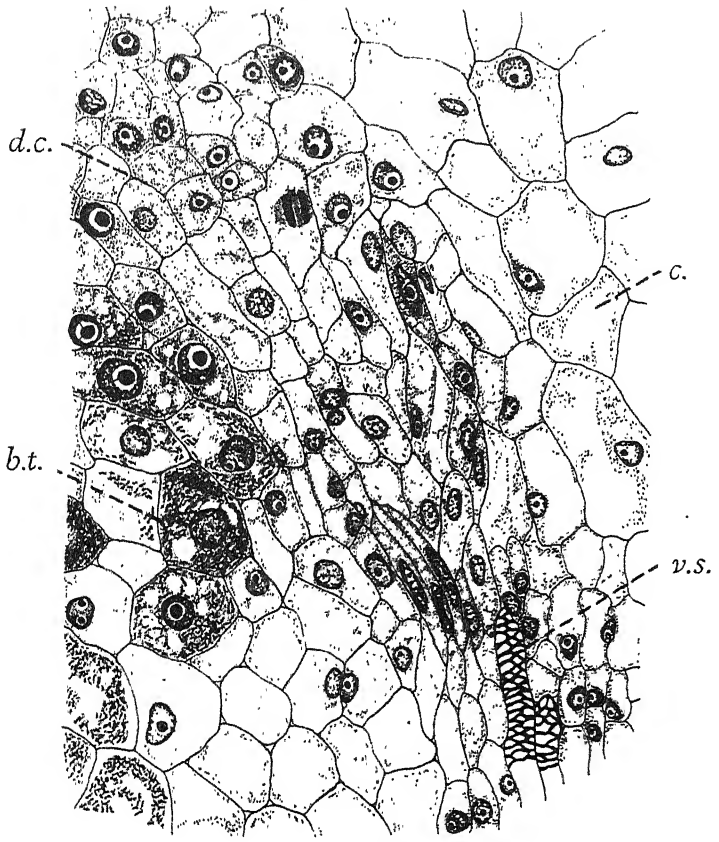

FIG. 12

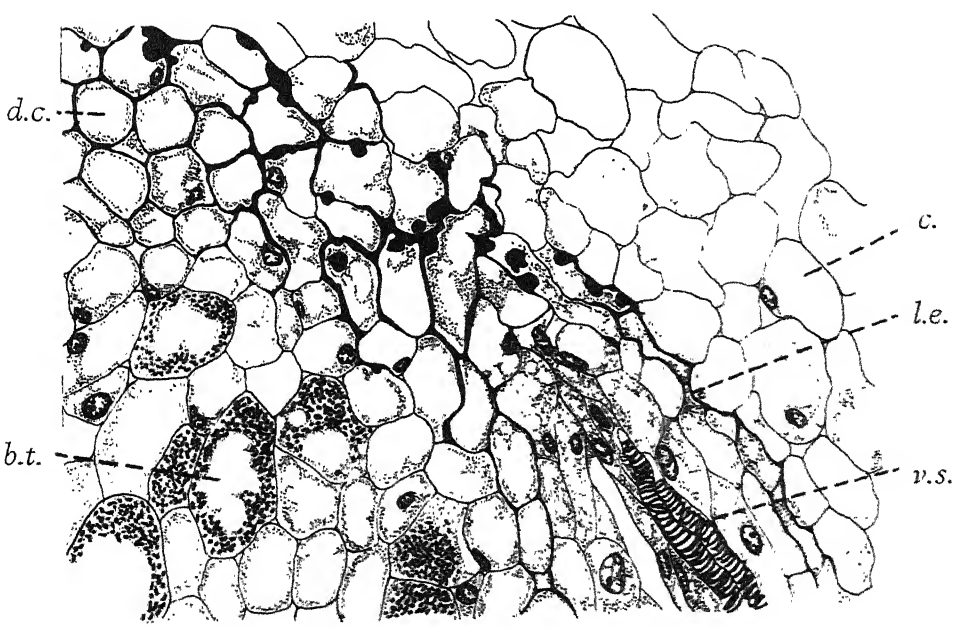

FIG. 13

595 · 792 *Microplectron*

Host Selection by *Microplectron fuscipennis*, Zett. (Chalcididae, Hymenoptera)

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I—Introduction

Microplectron fuscipennis, Zett., appears to be restricted for its host material to the European species of the Tenthredinid genus *Diprion*, Schrank. It has been recorded from *D. sertifer*, Geoffr., *D. pini*, L., *D. pallidus*, Kl., and, recently, from *D. polytomum*, Htg. The resting stage larva within the cocoon is attacked, oviposition occurring after the host has been paralysed.

The present paper is an account of part of a more extensive programme of work on *Microplectron* which is being undertaken in view of the promising nature of the parasite and of its introduction into Canada. The biology of the species has been dealt with by Morris and Cameron (1935) and its reactions to changes in the physical environment by the present writer (Ullyett, 1936).

The following account is of those initial studies which deal specifically with the manner in which the parasite finds and selects the medium upon which its progeny can develop to maturity.

II—A Review of the General Situation

By a consensus of opinion among workers who have devoted special attention to the subject, insect behaviour is denied the instigation of intelligence and is said to be guided by the promptings of instinct or by tropic responses to external stimuli. Thompson and Parker (1927) have defined the classical conception of instinct very clearly in the following words: " The purely instinctive operation takes place as if a judgment on the desirability or suitability of a given object had been formulated by the animal, although it does not possess either the data necessary for such a judgment or the mental equipment required for producing a judgment in the true sense of the word. In other words, it acts as if it were arranging things reasonably in relation to a given end, although it really cannot reason and can have no vision of the end ".

The selection of a host for oviposition by an entomophagous parasite presents a case which has given rise to some controversy in respect to the behaviour of the insect in this vital role. The maturation of ova within the ovaries provides a physiological urge which prompts the gravid female to seek a suitable medium in which, or upon which to deposit her eggs. She must not only seek the particular species of host which favours the development of her progeny, but she must also find the appropriate stadium in the life-cycle of the host. In her search, the female traverses a certain area within which she is bound to encounter many different objects, some of which may conceivably resemble the host in outward appearance. Furthermore, of the actual hosts which she discovers, some may be smaller than others; some may be parasitized already by females of her own or of a different species; others may be unsuitable for her progeny owing to death or disease. The powers of discrimination at the disposal of the female are limited, as far as we know, to instinct and to the reactions to sensory impressions aroused by the host in respect to shape, size, texture, and odour. It will be convenient to examine the literature to see how far the information already accumulated throws light upon the general problem of host selection.

1—*Factors Residing Within the Insect Itself*

The reasons assigned by various writers for the fact that a female insect is able to choose successfully the correct medium for oviposition can be classed under three main groups. In the first of these, the insect is regarded as being influenced mechanically by tropic responses. The ultimate reaction may be produced by a single stimulus; more usually it requires a chain of sensory impressions to complete the resolution of the initial impulse.

In the second group the view is held that inherent instinct provides the impulse which ensures that oviposition shall take place in the particular medium which is most suitable for the development of the progeny. Thirdly, we have those who contend that the nature of the medium upon which the female itself was reared is so imprinted upon the memory of the insect that this memory conditions the oviposition response in the desired manner. This view is not very far removed from the one preceding. At the present juncture, it is impossible for us to draw a distinction between instinct and memory with regard to insects. If we say that memory is involved, then this faculty admittedly produces a response as mechanical as that generally associated with instinct and we are, in reality, applying a different term to the same conception.

After a careful analysis of the data at their disposal, Thompson and Parker (1927) came to the conclusion that the choice of hosts is not a phenomenon which can be referred to any constant and definite efficient cause exterior to the organism, and that the acts of a parasite are not strictly determined if considered simply in relation to the physico-chemical properties of the host. In their opinion, the problem is a psychological one which can be understood only by comparison with similar phenomena in man as, for example, the choice of food, which can be examined by the process of introspection. From this point of view, the classical idea of instinct is the only one which appears to fit all the facts of the case.

On the other hand, we have a large school of thought in which the bias is inclined as strongly to the conception of tropic behaviour as the main determining influence in the choice of hosts. The conception necessitates our regarding the insect simply and purely as a machine which is actuated by this or that stimulus to perform certain well-defined movements (*vide* Loeb, 1918). The position from this aspect, with regard to the oviposition responses of insects, has been summarized by Richardson (1925). We shall consider here those physico-chemical properties of the host which may have an influence upon the searching female parasite.

2—*Significance of the Physico-chemical Properties of the Host*

Shape—The literature contains records of many species of polyphagous parasites which number among their respective alternate hosts, individuals of very varying shapes. Thus a single parasitic species may attack the chrysalids of certain Lepidoptera, the puparia of a dipteron, and the naked pupae of an hymenopteron; all of which are very different in outward appearance. It is difficult to conceive that shape is an important criterion in such cases, unless we are prepared to admit that the parasite is able to recognize and appreciate a number of different shapes which correspond to those of its several hosts.

Where the parasite depends upon a single species, or the individuals of a single genus for its host material, the case is different. Here, it is easier to see that shape may well prove to be a factor of importance in the decision of a female. The subject does not seem to have been studied very extensively from this point of view. The position with regard to *Microplectron* is investigated later.

Size—From purely theoretical considerations, it would be expected that the size of the host is important in guiding the female in her choice,

inasmuch as, other things being equally favourable, it is obvious that the larger the host the more likely it is that the parasite progeny will be able to develop successfully; or that more progeny will be able to develop on the individual host. There are two distinct cases for consideration, namely (*a*) where the parasite invariably deposits a single egg in each host, and (*b*) where the parasite progeny are gregarious. In the former, the size of the host is important so far as the size of each individual parasite is concerned. In the latter, the size of the host determines the number of progeny which each host will support to maturity.

Thompson and Parker (1927) have noted that, in general, the adults of parasites which are solitary in their habits, are usually about the same size as the adults of the host, or at least, not very much smaller; whereas there is a marked difference in size between the adults of gregarious parasites and those of their hosts. They further remark that it seems a general rule that the number of eggs normally deposited within a host is roughly proportional to the amount of food material available, *i.e.*, to the size of the host.

The same authors, in their study of the behaviour of *Melittobia acasta*, Walk., came to the conclusion that there is a limit below which hosts are inacceptable for the parasite and consider it probable that size is a determining factor in host selection in this species.

Recently, Salt (1935) has investigated the subject with reference to *Trichogramma evanescens*, Westw., a polyphagous egg parasite which is reared in the laboratory on the eggs of the Grain Moth, *Sitotroga cerealella*, Oliv. He compared the oviposition obtained in host eggs of different dimensions when these were offered to female parasites as definite alternatives and found that the larger hosts were almost invariably chosen for the reception of eggs in preference to the smaller hosts. From this, and from a consideration of results from other experiments, he came to the conclusion that size is the main criterion in the selection of hosts by this species. This preference is one which might reasonably be expected when a small egg, such as that of *Sitotroga*, is directly opposed to a larger one. It must be remembered that the Grain Moth egg is an *artificial* host of the parasite and that it is appreciably smaller than the smallest of the natural hosts of *Trichogramma*. In applying Salt's conclusion to distinctions between hosts under field conditions, we are faced with the difficulties produced by the artificiality of his experimental technique. It is pertinent to ask whether a female, which has traversed some considerable distance in search of a host, will reject one when found on the ground that, although one of her possible alternate hosts, it is smaller than the one she *might* find by journeying a little further over the plant?

Unless we can give a definite answer to this, any experimental results lack significance.

It is not necessary, however, to regard the problem of size as one which is primarily concerned in selection between two possible hosts. Although two or more alternate hosts of a parasite may occur on a plant at one and the same time this is likely to be a rare rather than a common occurrence. The actual significance of the dimensional character probably lies more in the limits it imposes upon the possibility of the parasite progeny developing within the host and in the capability of the female parasite of gauging this factor. A case which falls within the present writer's experience is that of *Trichogramma lutea*, Gir., which deposits a single egg in the egg of the Grain Moth (*Sitotroga*), whereas three or four may be laid in the larger egg of *Heliothis obsoleta*, Fabr. In both cases it is possible for all the parasite progeny to develop to normal maturity. The female parasite, therefore, must have a conception of size which is directly related to the welfare of the progeny, and we can understand that a lower limit of size, beyond which the parasite will refuse a host, is within the bounds of possibility.

Texture—It is well known that hymenopterous parasites subject their hosts to an examination before oviposition takes place. The visible manifestation of this inspection consists, as a rule, of a rapid and continuous tapping of the surface with the tips of the antennae. The latter organs are known to be the seat of both olfactory and tactile sense organs and hence this antennal exploration is capable of interpretation in either, or both, of two ways. In addition, there is the possibility of a simultaneous examination by the tactile organs situated upon the tarsi, and a final one by the sensitive tip of the abdomen or ovipositor.

As far as the texture of the host is concerned, we are again faced with a possible difference in reaction between polyphagous and monophagous parasites. In the latter, we might feel justified in attributing some measure of influence to the tactile impressions created by the host; but where a parasite is known to attack several different hosts, each with a very different integument, the idea is more difficult to visualize. There is no experimental evidence on this point to guide us to an opinion. At the same time, we cannot say what similarity might exist between the various hosts which, while not apparent to us, is readily discernible by the intensely sensitive tactile sensillae of the parasite. Furthermore, a polyphagous parasite may be capable of appreciating a number of different textures as readily as a monophagous parasite recognizes the single one peculiar to its host.

That the examination does concern itself in some way with the perception of texture becomes clear when we remember that, frequently, the parasite is known to oviposit along the line of weakness in the integument of the host. In all probability this is the true explanation of this aspect of behaviour and texture, of itself, is of secondary importance in the initial selection of the host.

Odour—The literature dealing with the chemotropic responses of insects has assumed vast proportions within recent years and our knowledge of this subject is very much more extensive than that of other aspects of insect behaviour. Most of the investigations recorded have been made from the economic point of view and concern the attraction exerted by volatile substances, many of which are derived from the odoriferous principles present in plants. It is well established that phytophagous insects are attracted to their host plants for feeding and oviposition by the characteristic odour emanating from the plant (*vide* the experiments of Verschaffelt, 1910, for example).

The sense of smell is also known to constitute the basis of recognition as observed between insects. Among the Formacoidea, recognition between members of the same colony, and between these and aliens, is accomplished by the perception of the odour-form of the insects (Forel, 1908). Males of Lepidoptera are attracted over considerable distances by the sexual odour of the females. Smell, therefore, plays a very prominent part in the life of an insect. It would not be surprising to find that it has an equally important role in the selection of hosts by parasites.

The location of the olfactory sense organs in insects has been the subject of considerable investigation by a number of workers. The general opinion is that these are concentrated on the antennae, although tarsal perception of food odours has been demonstrated in some butterflies and in certain Muscid flies (Minnich, 1921, 1926). A summary of the whole subject has been furnished recently by Marshall (1935).

The function of the olfactory sense in the selection of hosts by parasitic insects has not been investigated to any extent. With the powers at the disposal of an insect it might be supposed that the faculty plays an important part in this activity. With *Pimpla instigator*, F., an Ichneumonid parasite of the pupae of *Pieris brassicae*, L., Picard (1922) has shown that the females can be stimulated to performing the piercing action with the ovipositor by providing them with paper cylinders soaked in the blood of the host. The females continue to pierce the cylinders until the blood dries up but no deposition of eggs takes place. Picard concluded that the act of piercing is a " reflex action " determined by the odour of the

blood of the host but that actual oviposition depends upon a further stimulus provided by the contact between the ovipositor and the body of the host proper.

In a similar experiment, but using females of *Melittobia acasta*, Walk., Thompson and Parker (1927) were unable to confirm Picard's results.

In the same paper, these two authors, while admitting the possible importance of smell, have pointed out that, with species of parasites having polyphagous habits, the perceptible odours of the different hosts frequently vary greatly, both qualitatively and quantitatively. They refer, however, to the odour which is perceptible to man; but we cannot say that a common odour is not present—one which is readily recognized by the highly-developed sense of the insect, but which is not perceived by our own degenerated olfactory organs. It is not easy to see how this can be submitted to experimental investigation. Even a detailed chemical analysis of each individual host would hardly provide an argument for or against the possession of a common odour, even if the existence of a common principle were proved; as we do not know what effect the presence of other substances may have upon the odour of this principle, and what the combined effect upon the insect will prove to be.

From another standpoint, Salt and Laing (1935) have recently stressed the importance of smell in host selection. They state that, by means of this faculty, females of *Trichogramma evanescens* are able to distinguish hosts which have been visited by themselves or by other females of the species. This view is advanced as an explanation of Salt's results, where parasitized eggs were avoided in favour of those which were unparasitized. There is no indication as to how long this contact-odour effect will last, and we cannot say how long the parasitized egg will remain immune from further attack.

In the laboratory rearing of *Apanteles sesamiae*, Cam., the present writer (Ullyett, 1935) found that when the artificial burrows made for the host (*Busseola fusca*, Fuller) were fresh the parasite was not able to find the host larvae as readily as when some feeding had taken place, and a certain amount of frass had accumulated. This seems to indicate that smell plays a considerable part in the finding of the host by this species.

3—*Discrimination Between True and False Hosts*

It has been generally accepted in the past that parasite females are guided instinctively to deposit their eggs in the true host in an automatic manner. From its observed rarity in nature, any departure from this routine is regarded as an "error of instinct". The occurrence of the

latter has been compared by Thompson and Parker, to the case of a man who, under the stress of great thirst, drinks water which is polluted and which he would normally shun. It would seem that this view is true, or at least a close approximation to the truth; otherwise we should expect to find either (i) that high rates of parasitism were exceptional rather than common and that some species would tend to become exterminated through the wastage involved in attacking false hosts, or (ii) that a higher rate of reproduction existed among parasitic insects than is actually so in order to ensure the persistence of a species distributing its progeny at random. The question has been revived recently by Salt (1935), who, in his experiments with *Trichogramma evanescens*, found what he considered to be a definite preference for false hosts. He compared the relative attraction to the parasite of eggs of the laboratory host (*Sitotroga*) and such foreign bodies as grains of sand, pieces of glass, and Lobelia seeds. He records that, where the latter objects were larger than the true host, they were " attacked " in preference to the eggs and he presents a table giving the results of experiments in which the number of eggs laid by a female parasite was considerably reduced in their presence. From this he concluded that there was a definite preference for the false hosts due to their larger size, and that selection was not influenced by any " indefinable quality " emanating from the host egg. For the details of his technique and findings, reference should be made to the original paper. His work is open to some criticism from an experimental point of view.

In the first place, only the end results of experiments are presented. These are apt to prove misleading in cases such as the present. It must be remembered that the introduction of sand grains among host eggs provides an extra abnormality in what is essentially already an abnormal environment and that the reactions of the parasite may consist of something very different from actual preference for the intruding substance. The time allowed for oviposition was eight hours. This is relatively a small proportion of the total life time of the insect. Had the full period of adult life been allowed, it is possible that a smoothing out of the disparity between the true host series and those with alternated sand and eggs would have occurred. Females vary widely in the length of the pre-oviposition period, and while some oviposit early in life, others do so much later, and in the meanwhile will wander aimlessly or spend much time in examining both hosts and other objects with which they come into contact.

The main criticism of Salt's work, however, is that he did not eliminate entirely the influence of the host. We have seen that odour-stimuli play an important part in the life of an insect, and also that, in some cases at

least, the act of piercing is conditional upon this impulse. In an experiment where host material is confined in a small space, such as in a petri dish, we can conceive of a concentration of the odour of the host in the atmosphere within the space and the stimulus is presumably greater in its effects on the parasite under these conditions than where eggs are distributed in the open, as under field conditions where air movements are present. A good analogy is that of a small piece of orange peel which will impart its odour to the air of a whole room; whereas, when lying by the roadside it is not noticed. When sand-grains are placed in the same area and are alternated with host eggs, each grain is surrounded by the latter and may come within the sphere of the odour-stimulus emanating from each of these eggs. Any "attack" on the sand-grain, therefore, might be attributable to this stimulus and not to any actual preference for the sand-grain on account of size alone. It is extremely difficult to eliminate this odour-stimulus of the host. It is more than probable that newly emerged adult parasites retain some vestiges of the odour acquired through contact with the host and that this, under confined experimental conditions, may be sufficiently strong to contaminate any object with which they meet or to taint the atmosphere in such measure as to produce the appropriate reflex action upon encountering an object. In such experiments, it would be better, perhaps, to use females which have been isolated from any host material for some time following emergence, rather than to use ones which are newly emerged.

Considered from a purely logical standpoint, it seems incredible that the female should waste both time and energy in endeavouring to oviposit in an impenetrable object in the presence of suitable hosts, provided that conditions are such as can be counted natural. We cannot conceive of this happening in the field; otherwise we must recognize that the innumerable objects encountered during the wanderings of the female would contribute so largely to failure in discovering the true object of her search as to result, virtually, in the extinction of the species.

4—*Distinction Between Parasitized and Unparasitized Hosts*

Assuming that a female parasite is capable of distinguishing between its host proper and other objects which are totally unsuited to the development of her progeny, the following two questions arise: (i) Can the female discriminate between a host which is bearing parasite progeny of the same species but of a different female, and one which is as yet unparasitized? (ii) Is she able to recognize a host which she herself has parasitized previously, but which she has rediscovered after leaving it for a time?

If we assume that no such distinctions can be made, then an ovipositing female will deposit her eggs in host individuals as these are encountered in her tour of inspection; and as this will happen by chance, the eggs will exhibit a random distribution among the host population. This has been the generally accepted opinion up to the present time. We shall examine the evidence for and against this theory.

After considerable experience with the parasites of the Gipsy Moth (*Porthetria dispar*, L.), Fiske (1910) found that, while a few species exhibited faint indications of discretionary powers, the vast majority of the parasites studied by him showed no such perception. Oviposition, therefore, occurred at random with a consequent incidence of superparasitism. On the basis of these observations he constructed curves showing the effect of this phenomenon on the parasite population.

On the same assumption, that the theory of random distribution of progeny is correct for the majority of parasitic insects, Thompson (1924) investigated the subject mathematically and produced the formula:

$$Y = N(1 - e^{-\frac{x}{N}}),$$

where Y is the number of hosts parasitized, N the number of hosts in the initial population, x the number of parasite eggs, and e the Napierian logarithmic base. In later works, this author developed formulae to cover special cases.

It must be understood that this mathematical treatment has been based solely upon results of field observations and on the examination of numerous samples collected from the field. It is the expression of a generality derived from many different species. To the vast majority of such similar records as are available, the degree of approximation given by these formulae is reasonably good. The formulae, however, were not subjected to the ultimate proof afforded by experimental methods.

Apparently the first attempt to provide laboratory data of this nature was that of Trouvelot (1923), who carried out a series of population studies with *Habrobracon johannseni*, Vier., an ectoparasite of *Phthorimaea operculella*, Zell. These were designed to show the incidence and effects of superparasitism with varying densities of host population. His results, however, do not go sufficiently far to give us the requisite data for our present problem.

Working with *Melittobia acasta*, Walk., Thompson and Parker (1927) record that the females will not oviposit in puparia of Diptera which contain either their own larvae or pupae, or those of *Pteromalus* or *Dibrachys*.

The problem has been more recently attacked by Salt, whose attention was first arrested by an apparent discrepancy in the behaviour of *Collyria calcitrator*, Grav., an Ichneumonid parasite of *Cephus pygmaeus*, Linn. (Salt, 1932). He found that the data obtained from numerous dissections of *Cephus* larvae did not agree with the values to be expected from the random distribution of parasite eggs, and that a degree of discrimination on the part of ovipositing females was indicated, which led to their avoidance of hosts which were already parasitized. It must be emphasized, however, that this discrimination was by no means absolute. Furthermore, Salt does not indicate in what manner his samples for dissection were obtained—an important consideration when dealing statistically with material collected from the field, where parasite activity varies, not only with climatic conditions, but from place to place within a prescribed area (*vide* Parsons and Ullyett, 1936).

In his more recent work, Salt (1934) has examined the problem from an experimental point of view, choosing as his subject a common egg parasite. From his results he concluded that *Trichogramma evanescens* is not only capable of discriminating between host individuals which are already parasitized and those which are not, but that it habitually does so, and that, therefore, its progeny do not follow a random distribution among the host population. As an explanation it has been suggested (Salt and Laing, 1935) that recognition of a parasitized egg is by means of the contact-odour left on the host by the previous female.

These conclusions are supported by the work of Lloyd (1935) on *Ooencyrtus* (*Schedius*) *kuvanae*, How., a Chalcid egg parasite of *Porthetria dispar*, wherein he found a discrimination of a high order. In addition, a certain degree of restraint in oviposition appeared to be exercised by females when in contact with previously parasitized material—a point also noticed by Salt with *Trichogramma*.

The application of these very striking results to the interpretation of field observations seems, to the present writer, to demand a considerable degree of caution. Before bringing them into the realm of practical biological control as established facts, it would be advantageous to know something more about the relation between experimental and field conditions than we do at present. If a field case is examined the issue will not be clear. Quoting an example with which the writer is familiar, we may investigate the position of *Trichogramma* when released in, say, a maize field. For the sake of simplicity we shall assume that she alights directly upon a plant which is normally well grown. When the host population is small there may be three or four eggs distributed over the

plant, and the female will almost certainly be faced with the necessity of traversing a very considerable area in search of the first host. By the time she has discovered it, some hours may have elapsed since she emerged and mated. The next host is equally remote from the first. At the other extreme a fairly heavy host infestation on a maize plant would be represented by a total of 30 to 40 eggs which, when spread out over the plant, are by no means conspicuous or closely massed. The female still has a considerable amount of searching to perform and in the course of this she is likely to encounter a number of host individuals which have been parasitized by other females. Bearing in mind the fact that distances between hosts are great in relation to the size of a parasite, can we conceive of the female exercising "restraint" and actually making a deliberate choice from among a widespread host population?

It is to be observed that in our field example conditions are very far removed from the laboratory experiment. In the latter we have host eggs in close proximity to one another and supplied in adequate numbers; in the former, the hosts are scattered over relatively large areas and are irregularly distributed not only as regards each individual plant, but also as between different plants within a field.

5—*Discussion*

In the foregoing review no attempt has been made to produce an exhaustive summary of the existing literature on host selection. It is hoped, however, that sufficient has been put forward to give a general idea of the present extent of our knowledge on the subject and to provide a background for the experimental work to be described.

The modern trend of thought is removed from the old conception of instinct as the sole basis of insect behaviour and the latter is coming to be regarded as the natural outcome of a chain of impulses created by external stimuli of various kinds. At the same time the position is far from static and there is a vast field open for research, particularly with respect to host selection by entomophagous parasites. Our information on the latter is meagre in the extreme and, as we have seen, the experimental work already accomplished is either open to criticism or is difficult to reconcile with field conditions. It is in this aspect of the subject that intensive investigations are urgently required, especially with regard to the effect of abnormalities which are inevitably introduced into laboratory experimental work.

We have yet to define and determine the effects of abnormal environments. It seems not unlikely that the majority of our laboratory experi-

ments, however closely they appear to conform to what we consider optimum conditions for the insect subject, may contain some abnormal element of which we know nothing but which, nevertheless, duly affects the delicate organism with which we are dealing. We may cite, as an example, the frequent instances which occur of parasitic insects refusing to mate or breed under laboratory conditions; the underlying reason for this behaviour is usually obscure.

As to whether a parasite is able to exercise any power of discrimination between parasitized and unparasitized hosts, opinion is divided. This power appears to be present in some species, at least under laboratory conditions, but it is obvious that we cannot generalize on this point and we cannot say, as yet, what significance this has in relation to field problems. On the other hand it seems that, in general, the female parasite has some conception of size and that she will choose a host which is large enough to support her normal complement of progeny to maturity.

It is difficult to reconcile the phenomenon of host selection with a purely mechanistic explanation. The true solution seems rather to be in a compromise between the psychological and tropic views. In so far as certain odours attract a particular insect, these must be imprinted on the "mind" of the insect in such a way that a mechanical response is produced in their presence. The same applies to the other attributes of the host. It is most probable that selection depends upon a pattern of impressions rather than upon a single factor, and that each of these impressions forms part of a mosaic sense-picture, which is ever present in the "mind", which is hereditary and which, in reality, provides the basis of instinct.

Our knowledge on host selection among entomophagous parasites is by no means comparable with that available for phytophagous insects. Much of the literature deals in mere surmise or in theories founded on relatively few facts. From the nature of the problem this is inevitable and, until our experimental technique has developed and improved, we cannot hope to extend our knowledge appreciably.

III—The Nature of the Present Problem

Before proceeding to an account of experimental work on host selection by *Microplectron fuscipennis*, it will be convenient to review the known, relevant facts concerning the species as gathered from field records and from laboratory observations.

As far as is known, *Microplectron fuscipennis* is restricted for host material to the single Tenthredinid genus *Diprion*. The actual host is

the resting stage larva of the sawfly, which is enclosed in a compact, fibrous cocoon. Its presence, therefore, can be detected by the parasite only by means of the possible tactile function of the ovipositor or by chemical stimuli acting on the olfactory receptors. It is also possible that the association of the particular type of cocoon with the host has some significance in this respect; although Thompson and Parker (1927) came to the conclusion that, with *Melittobia acasta*, " the behaviour of the parasite does not depend so much on the precise physical characteristic of the envelope surrounding the host as on what lies beneath it ". The larvae of the different species of sawfly attacked present a similar appearance, are of approximately equal dimensions, and all possess a similar, strongly perceptible odour reminiscent of the food plant. It is possible, therefore, that *Microplectron* may be regarded as possessing the virtual status of a monophagous parasite.

The progeny are gregarious and a single host may support up to nearly 100 parasite larvae, which will give rise to normally functioning adults. In excess of this number, the resulting parasites are dwarfed and incapable of reproduction. Each female parasite will attack only one or two hosts for oviposition and may deposit all her eggs in a single cocoon. As the number of eggs produced by an average female is between 50 and 60, the host is not only fully capable of supporting the total progeny of two separate females, or perhaps more, but does so very frequently.

As the oviposition and general activity of the parasite are closely related to atmospheric conditions, all experiments must be conducted at a constant temperature and humidity, lying within the zone of optimum conditions for the parasite (Ullyett, 1936). At each temperature the female takes a certain time in which to paralyse the host and deposit the first eggs, and this period must be computed if a visible end result is required.

It is clear from the foregoing, that the investigation of host selection by *Microplectron* is complicated at the outset by the nature of the host material and by the oviposition habits of the female. In the following work, attempts to overcome these difficulties have been made with varying success. As the present investigations form part of an essentially practical study of the parasite, an endeavour has also been made to keep the experimental technique and the interpretation of results as near as possible to the boundary between laboratory and field conditions.

IV—Factors Influencing the Selection of the Host

1—*The Shape of the Host*

The hosts of *Microplectron* are all characterized by a similar shape; consequently this peculiarity might well be an important guiding factor. The following experiments were made with a view to obtaining definite evidence on this point.

In order to obtain unbiassed results, it was necessary to eliminate any other quality of the host which might influence the selection by the female. The external shape of the normal host is simulated very closely by pharmaceutical gelatine capsules. It was also possible to secure a capsule approximately the same size as the cocoons of *Diprion polytomum*, upon which the parasite had been reared in the laboratory. These were used in place of the normal host.

Four gelatine capsules and four rectangular pieces of pith of the same dimensions were alternated on a card which was placed in a glass tube. Four freshly-emerged females were then introduced and their behaviour noted. This observation was repeated five times in all, using fresh females from a different stock thus giving records of a total of twenty females.

The results in every case were in absolute agreement thus making it unnecessary to extend the series of observations with these particular objects. The females examined both the capsule and the pith, but the reaction in each case was sharply differentiated. With the pith the antennal examination was comparatively short and continued only until the sharp edge of the pith was discovered. After this occurred, no further interest was taken in the object and the female walked off and away. When a capsule was encountered, however, the examination was prolonged and covered the whole of the object. Special attention appeared to be centred in the sides and ends of the capsule. Finally, the females settled down to repeated attempts to pierce the gelatine.

The experiment was amplified by two further series, in both of which gelatine capsules were still used to simulate the natural shape of the host. In the first of these series, square pieces of pith were substituted for the oblong ones. In the second series, appropriate lengths of twigs of the same diameter as the capsule and with squarely-cut ends were used. In both series the number of observations made was the same as in the original experiment. The results obtained confirmed those previously given in every particular.

It seems clear that females of *Microplectron* have a conception of the shape of the normal host and that they are able to ascertain the shape of an object by means of the antennal exploration. The rejection of objects which are not completely rounded to resemble the cocoons of *Diprion* indicates strongly that shape, in itself, is an important factor in the selection of a host by this species.

2—*The Size of the Host*

Eight gelatine capsules of the same size as the normal host of the parasite were alternated on a card with eight capsules of a larger size (No. 3) and the card was placed in a petri dish. Eight freshly-emerged females of *Microplectron* were introduced into the dish and thus were confronted with a definite choice between small and large objects, both of the correct shape but lacking all other characteristics of the host. The females were kept under observation for three hours. Six series of such observations were carried out, giving a total of 48 females in the experiment.

Of these 48 females, 26 attempted to pierce the large capsules with their ovipositors, while only four gave a similar reaction to the small capsule. There was thus a definite preference for the larger object under the conditions of the experiment. This aspect of the subject will be considered in § 5.

3—*The Visibility of the Host*

The powers of vision in insects are admittedly limited. In the most feeble cases, however, the compound eye is able to distinguish, at least, between degrees of light and shade. An opaque object, on account of the denser shadow which it casts, is more easily discerned from a little distance than one which is transparent. As transparent capsules were used largely to simulate the host of *Microplectron*, the following experiment was carried out to ascertain how far this factor of visibility is important in the discovery of the host by the female parasite.

Five gelatine capsules were filled with brown paper and arranged on a card in a petri dish. From a number of females which had been isolated from host material for 48 hours following emergence, five females were taken at random and were introduced into the dish. Release was made in the lid of the dish and the parasites were left to find their own way down to the card bearing the capsules. The females were observed continuously for three hours, during which time particulars of their behaviour were noted. This series was contrasted with a simultaneous

series, similarly arranged, but with empty capsules. All characteristics of the normal host, therefore, were absent with the exception of shape and size. For the purpose of the present experiment, the ultimate reaction was taken to be indicated by the piercing action of the female. The series were repeated eight times, giving a total of 40 females for each. A fresh stock of parasites was used each time. The results obtained are summarized in Table I.

TABLE I

Series	No. of ♀♀	No. of examinations	No. of reactions	Examination to reaction Time in min.
A—Empty capsules	40	13	5	7·4
B—Capsules filled with brown paper	40	16	12	7·5

Where the females were confronted with empty capsules, only 5 out of the 40 gave a positive reaction. Apart from these isolated examples, very little interest was taken in the objects supplied. In most cases where females crossed the card, they walked past the capsules without deviation from their original route, even though a faint shadow from the object fell across their path.

When the capsules were filled with brown paper, much more interest was evinced for the objects and more, really definite visits were made. These occurred mainly when the shadow of the capsule crossed the original route of the female. This shadow was well defined and very plainly influenced the direction taken by the parasite when the latter entered the area affected. In all, 12 out of 40 females gave a positive reaction during the period of the experiment.

From the above, it is safe to assume that the degree of visibility possessed by the object has a direct bearing upon the ease with which the host is discovered. At the same time, it is not possible to consider visibility as a major factor in host selection. If this were so, the numbers reacting in the more visible series would be higher and the rate of reaction, under the conditions of the experiment, would be greater than was actually recorded (*cf.* later experiments where the normal host was used).

4—*The Texture of the Host*

The nearest semblance to the external texture of the host cocoon obtainable was that afforded by brown paper, which was lightly glazed on one surface. Capsules of the requisite size were covered with such paper as smoothly as possible and were exposed to females as in the previous

experiment. These were contrasted with a series in which bare capsules were filled with the same paper in order to give a comparable visibility. The characteristics of the host which were common to both series were therefore shape and size only. Observations were continued for three hours and a total of 20 females was used.

The results obtained from this experiment were inconclusive, four females giving a reaction with the bare capsules and three with the paper-covered ones. An explanation probably lies in the difficulty of reproducing the actual texture of the host. Although the brown paper used was apparently near the latter in outward appearance, we cannot say that, to the parasite, it bears any more resemblance to the texture required than does the surface of the capsule itself. It is significant that both are glazed, as is the surface of the normal host. Furthermore, the covering of a capsule entails the making of joints and creases in the paper, which are bound to be discernible to the parasite and, to this extent, affect the important factor of shape.

Texture, if anything, would appear to be of small importance in the initial selection of the host. This is borne out by a comparison with results obtained in other experiments. If it were a primary guiding factor, we should expect any reaction to be very much slower in its absence than actually occurs.

5—*The Odour of the Host*

When ten cocoons of *Diprion polytomum* and ten gelatine capsules of the same size were alternated on a card and exposed to the attack of ten female parasites, a complete reaction was obtained in eight cases, while in nine cases an antennal examination occurred. In all of these, the females were attracted directly to the cocoons and the capsules were ignored entirely. The former, therefore, clearly possess some influence which causes them to become the centre of a powerful attraction. The most reasonable supposition seems to be that this influence is of a chemotropic nature. To obtain definite information on this point, the following experiment was carried out.

Five gelatine capsules were mounted on each of two cards. On one of these a small smear of host blood was made next to each capsule. This smear was kept fresh throughout the experiment. The cards were placed in two petri dishes and five females of *Microplectron* were introduced into each, release being made in the lid of the dish. The females used were 72 hours old and had been isolated from host material during that time and had been fed on raisins. Observations were made con-

tinuously for two hours under uniform environmental conditions. Three such tests were made, giving a total of 15 females under observation. The uniformity of results precluded the necessity of further repetitions. The behaviour of the females is summarized in Table II, but a more complete picture is obtained from the following running commentary which was made at the time.

TABLE II

	A Capsules and blood smear	B Capsule alone
Total females	15	15
Antennal examinations of capsules ..	6	3
Complete reactions (piercing)	2	1

Series A (Capsules and blood smears)—Throughout the experiment, great interest was evinced in the blood smears. Females, when walking over the card, orientated in the direction of a smear when approximately 4 to 5 mm distant from it, after which an examination of the smear took place and, in a number of cases, this was followed by the mounting of the capsule. In all cases, any approach to the capsules was invariably *via* a blood smear. During the time of the experiment, all the females visited the smears, although less than half examined the capsules. Examinations of capsules lasted, on an average, for half a minute and were, therefore, very brief. The explanation for this and for the lack of complete reaction in all but two cases must be sought in causes other than odour alone. It is noteworthy that interest in the capsules was more intense on the side which faced the smear. At the end of the experiment, the blood was allowed to dry, after which no further attraction of the females occurred.

Series B (Capsules alone)—The small number of examinations reflects the general lack of interest exhibited. When the capsules were encountered during a traverse of the card, females usually walked over them and very obviously treated them as mere obstacles to be surmounted. There was no definite centre of attraction as was manifest for the blood smears. The mounting or examination of capsules occurred purely after chance encounters by the females concerned.

Odour is clearly of importance in the initial attraction of the female parasite to the host from a certain distance. There is evidently a sphere of attraction surrounding the host which is determined by the emanation of the characteristic smell of the insect. In the present case, the blood

smear exerted this attraction over a distance of 5 mm as shown by the orientation of the females. The area of attraction is undoubtedly greater with a normal and perfect host insect. The odour of the host of *Microplectron* resides primarily in the body of the larva and is present in the blood. We shall have further opportunities of assessing the value of this characteristic.

6—*Discrimination Between True and False Hosts*

Earlier in the present paper, it has been suggested that the alternation of true and false hosts on a single card within a confined space is a method which is not sufficiently reliable to give a true measure of the relative attractiveness of the two objects. The influence of the normal host, as regards its odour, is not entirely eliminated where the false host is concerned and may affect the reactions of the parasite to the latter. In studying the problem it became necessary to find an alternative method which was free from this objection. The final experimental technique was based upon the following reasoning.

Assuming that the true host possesses all the characteristics which, in combination, make up the sense picture present in the " mind " of a female parasite then a given number of females might be expected to exhibit a greater rate of reaction to the normal host than to a false one. Also, they might be expected to show a greater number of reactions to the normal host within a given time. Similarly, when two false hosts are compared, then the one having the greater number of the characteristics of the true host might be expected to exhibit a greater attraction and to produce more reactions than the other. If, then, a series of observations are made in which the members of the series are dealt with individually, but at the same time, under the same physical environment, and using equivalent numbers as regards both females and objects, a record of the number of complete reactions obtained throughout a given time should indicate the relative attractiveness to the parasite of each member of the series. In this way, the influence of the normal hosts is eliminated from the environment of the false hosts. Furthermore, the individual members of the latter series can be arranged in such a way that the separate properties of the normal host can be investigated in relation to their respective importance in influencing the female parasite.

In the light of previous experiments, any object not possessing the general shape of the host was ruled out. False hosts were represented, in each instance, by gelatine capsules. In order to obtain some idea of

the importance of the characteristics involved, as well as information on the general problem, the following series were arranged:

Series A—The normal host—cocoons of *D. polytomum* containing resting-stage larvae.
Series B—Gelatine capsules, each containing a larva of *D. polytomum* which had been removed from its cocoon.
Series C—Empty cocoons of the host.
Series D—Empty gelatine capsules.

The capsules used were of the same size in each series and were of approximately the same dimensions as the true hosts. In every trial, five of the objects in each series were arranged in the same manner on a card which was then placed in a petri dish. Each object was treated

TABLE III

Characteristic	Object			
	A Normal host	B Capsule and larva	C Empty cocoon	D Empty capsule
Odour	+ +	+	+	−
Shape	+ +	+ +	+ +	+ +
Size	+ +	+ +	+ +	+ +
Texture	+ +	−	+ +	−
Visibility	+ +	+ +	+ +	−
Larval movement	+ +	+ +	−	−
Expected order of attractiveness	1	2 or 3	2 or 3	4

separately in its own dish. Five females selected at random from a population which had been isolated from host material for 48 hours following emergence, were introduced into the lid of each dish, series A to D being commenced simultaneously. The females were observed continuously for three hours. The numbers giving a reaction in each dish during the time of the experiment and the time at which each reaction occurred were noted.

Before giving the actual results obtained in the experiment, it will be useful to consider how the various objects compare with regard to the characteristics of the host which are present or absent. This comparison is made in Table III, where ++ denotes the complete presence of the characteristic, + its partial presence, and − its absence. The expected order of attractiveness to the parasite, based upon the total characteristics possessed by each object, is shown at the foot of the table.

In series B, the gelatine capsules used were not so porous as the host cocoons and the odour from the contained larvae would not be so readily perceptible from the exterior as in the case of the normal host. For this reason the series is credited with partial presence of the characteristic.

Each series of observations was made with females selected from a different stock population and the observations were made at different times, although in a similar environment. Under these conditions, each set of observations gave similar results and it was not necessary to extend the experiment beyond the eighth repetition. This gave a total of 40 females in each series. Their behaviour is summarized in Table IV.

TABLE IV

	A Normal host	B Capsules and larvae	C Empty cocoons	D Empty capsules
Total females	40	40	40	40
Number of females making examinations	37	33	37	13
Number of females accepting the object	37	27	6	5
Time in minutes; examination to acceptance	4·6	6·4	10·6	7·4
Order of attractiveness	1	2	3	4

The trend of events is clearly indicated by the figures given, but these are amplified by notes made during the course of the experiments. Thus, in series D, the figures show that a very small proportion of the females evinced an interest in the objects. Apart from these isolated examples there was an entire lack of special interest in the capsules. Where females encountered these in their traverse of the card, they were treated merely as an obstruction and, although mounted, were never subjected to any antennal examination. Furthermore, where examination of a capsule did take place, this was always preceded by a chance encounter with the object and did not occur as the result of any definite attraction. In the majority of such cases the female concerned had already encountered one or more capsules prior to the one which she ultimately examined. The significance of this particular point will be discussed later. For all practical purposes, the results in this series may be taken as showing a complete rejection of the capsule as a host.

In series C, there was very great interest in the empty cocoons, as shown by the number of females which submitted them to an antennal examination. There was a definite attraction for the female, this initial stimulus being most probably an olfactory one. The rejection of the object as a suitable host was almost as complete as with the capsules.

Series A and B, on the other hand, show a high degree of attraction and acceptability as a host, the latter series being only a little inferior to the former in this respect.

A comparison of the four series is made graphically in fig. 1.

It is clear that a very definite discrimination between true and false hosts is exercised by the female. This is evident when series A and B are compared with series C and D. In series C the empty cocoon is essentially a false host inasmuch as the larva, necessary for parasite development, is absent. While it retains those characteristics of the host which provide

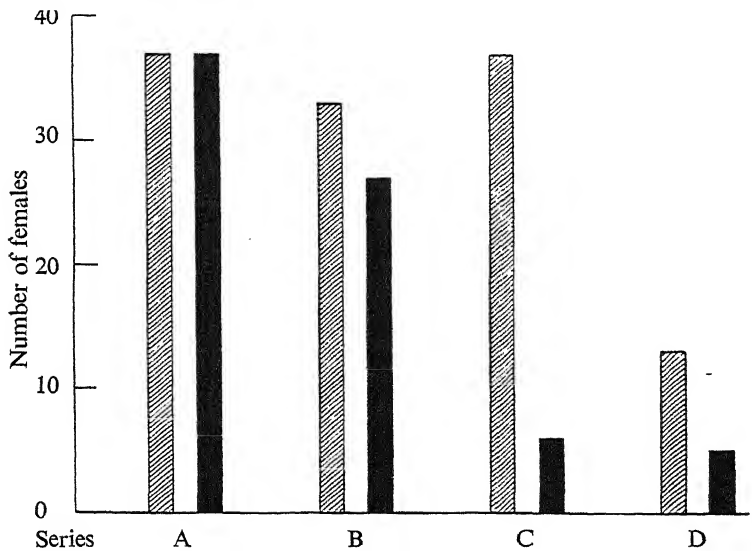

FIG. 1—*Microplectron fuscipennis.* Responses of 40 females to different objects offered as hosts. Series A = the normal host; series B = gelatine capsules containing larvae; series C = empty cocoons; series D = empty gelatine capsules. *Shaded bars* show the number of females examining the object; *black bars* show the number of females accepting the object.

the initial attraction for the female parasite, the ultimate acceptance of the cocoon as a host is precluded owing to the fact that no medium for oviposition exists below the surface and that the parasite is able to perceive this without any preliminary use of the ovipositor. It will be observed that the order of attractiveness actually given agrees with that to be expected from an analysis of the properties of the objects, as given in Table III.

We can now consider the relative importance of each of the characteristics possessed by the true host in their role of guiding the female parasite to a choice.

It seems that the most powerful initial attraction is that exerted by the *odour* of the host. This was emphasized in the previous experiment where blood smears were found to effect an orientation on the part of the female. In the present series it is shown by the behaviour of the female in series A, B, and C, as contrasted with that in series D. In the former, well-defined attraction and orientation was manifest and is reflected in the number of definite visits paid to the objects and in the antennal examinations which occurred as a result of the attraction. On the other hand, the absence of odour in series D was accompanied by a lack of any definite initial orientation in the direction of the objects and the latter were encountered purely as a matter of chance. By itself, however, odour does not produce the complete reaction which indicates acceptance by the parasite. In this the present case differs from that recorded by Picard (1922).

Texture, while it may be of some significance in other aspects, has no important influence on the final decision of the female as is shown by a comparison of series A, B, and C.

A previous experiment has indicated that the *shape* of the object acts as a guide to the female in her initial examination and that this character is a definite element in the pattern of sense impressions received by the female. Under normal conditions, however, it is clearly unable to induce a final acceptance of an object which is devoid of other peculiarities of the normal host.

Size is of no importance in the distinction between hosts and foreign bodies and has no bearing upon their ultimate acceptance or rejection.

Visibility is only of significance in aiding the parasite to locate an object which has already exerted an attraction by means of smell.

It would seem that the ultimate acceptance of an object as a host by *Microplectron* depends upon the *presence of a living larva* beneath the covering envelope. This appears to be the only feasible explanation of the great difference exhibited between series A and B on the one hand and series C on the other hand. It is the only feature of the normal host which is missing from the latter series but which is present in both of the former. It is reasonable to assign the significance of this fact to the perception by the parasite of *larval movements*, such as, for example, the rhythmical heart-beat and the movements of respiration, of the mandibles and of the muscles. The importance of this factor is further emphasized by the comparison made in the chart in fig. 2.

It should be mentioned here that if females of *Microplectron* are offered empty gelatine capsules, as in series D, and observation is prolonged for a sufficient length of time as, for example, over a period of 24 or 48 hours,

acceptance of the object as a host may occur with a much larger number of individuals than is recorded in the experiment above. The writer is of the opinion that this cannot be taken to mean that the object is normally acceptable to the parasite. The following is offered as a reasonable hypothesis to account for this behaviour.

The empty capsule possesses only two of the perceptible characteristics of the normal host, namely, shape, and size. Of these, shape has been shown to be important in the initial stages of host selection. By extending

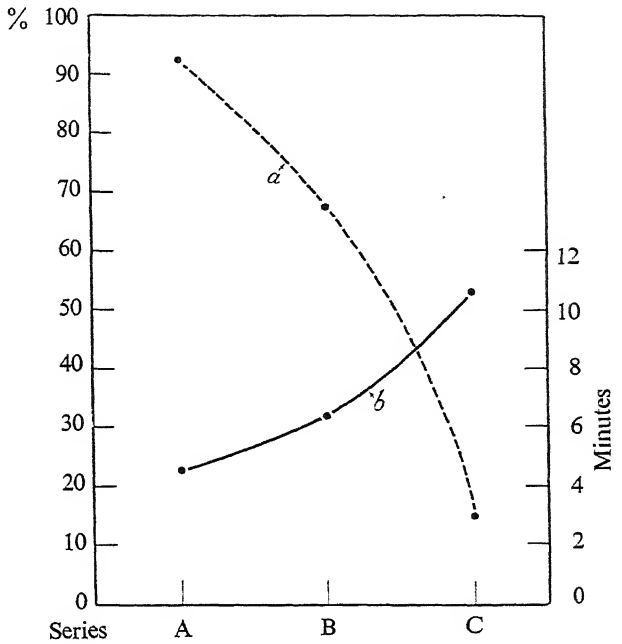

FIG. 2—The effect of larval movement on the selection of the host as reflected in the percentage of females accepting the object offered and in the time taken for them to do so. Series A = the normal hosts; series B = gelatine capsules containing host larvae; series C = empty cocoons of the host.

the time of the experiment to beyond the point at which a normal host is accepted, an abnormality is introduced into the technique. In effect, the female parasite is being confronted continuously with the single characteristic of shape, which admittedly forms one element in the pattern of sense impressions leading to acceptance of the host. For a time she rejects the object, after antennal exploration has revealed no further peculiarities which can be recognized. Under field conditions the parasite would then leave the object and continue the interrupted traverse of the surrounding area. A further meeting with the same or a similar object

would be unlikely to occur immediately and, indeed, might not happen at all before encountering the true host. In the laboratory experiment, where a small area is traversed, and where a relatively short distance exists between the objects offered, successive encounters are being made continuously with a number of similar objects. Thus, the rejection of a capsule is followed, almost immediately, by an encounter with its duplicate. In this way the isolated character of shape is being presented to the insect continuously and, although rejection of the object takes place for some time, this constant harping upon one theme may give a fictitious importance to the one element present, which ultimately leads to the "error of instinct" exhibited in the acceptance of the object as a host. The satisfaction of the physiological urge for oviposition is a powerful element in the reaction of a parasite to its host. The forcible inhibition of oviposition, therefore, may increase the acceptability of an unsuitable object after the lapse of a certain time.

Whatever the true explanation, it would seem that time is a factor of importance in experiments of this nature and should be carefully computed before observations commence. If an experiment is too extended, the significance of the end results is apt to become obscured. The criterion is, obviously, the time required by a parasite to find and accept the normal host under the conditions of the experiment. This principle was accepted in the three-hour experiments recorded above.

V—Selection Within the Host Species

It has been shown that the female of *M. fuscipennis* is able to discriminate between true and false hosts. Under normal conditions the true hosts themselves, however, do not present a homogeneous population. In the first place, a certain percentage of the individuals are smaller than the average of the whole population. Secondly, individuals occur which are not suitable for the development of the progeny of the parasite. This may be due to (*a*) the death of the host larva; (*b*) to previous and adequate parasitism; or (*c*) to advanced development of the host. In all cases, the true nature of the host is obscured from external examination by the presence of the enveloping cocoon. The female is faced, therefore, with a choice between healthy and unsuitable host individuals as well as one between hosts of different size. It is our purpose to endeavour to find whether the parasite is able to exercise any discrimination and to examine the means, if any, which are employed in the process.

1—*The Size of the Host*

In making a visual comparison, there is a very noticeable difference in size between large and small cocoons of *Diprion polytomum*. Actually there is a disparity of 1 to 2 mm in length and a proportional decrease in the girth of the small cocoons. Two series of experiments were carried out to determine the significance of this dimensional factor in the selection by the parasite.

In the first of these, large and small cocoons were alternated on a card which contained a total of 25 host individuals at regularly spaced intervals. The card was placed in a petri dish and an equal number of females was introduced. In the second series, the same number and arrangement of

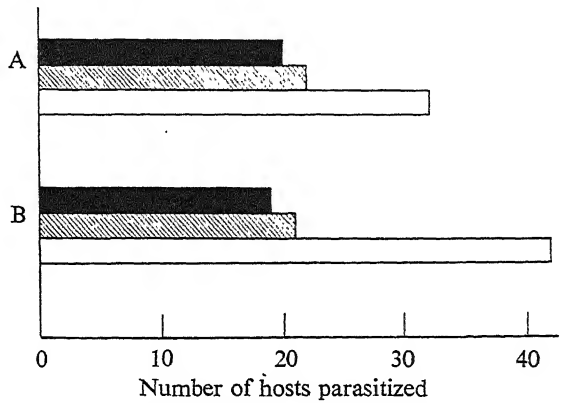

FIG. 3—Size of host and host selection: length of exposure. Series A = hosts exposed to female parasites for 24 hours; series B = hosts exposed for 96 hours. Number of hosts parasitized by 50 females in each series. Black bars = small cocoons; shaded bars = large cocoons; plain bars = control containing all large cocoons.

cocoons was followed, but each female was given a definite choice between a large and small cocoon. With each series a control, consisting of all large cocoons, was carried out at the same time in order to maintain a check upon the total parasitism. The series were left in an incubator at 25° C and 70% relative humidity for 48 hours, when they were removed and the cocoons examined for parasitism.

The period allowed for oviposition was based on the time normally required by a female to complete her oviposition in the first host under the conditions of temperature and humidity chosen (*vide*, Ullyett, 1936). In order to test the validity of this factor two trial series were arranged using a total of 50 females in each. Of these, one series remained undisturbed for 96 hours, while the other was removed after 24 hours' exposure. The results are shown in fig. 3 and reveal practically no difference between

the two series, from which it was concluded that the 48 hours' exposure was a suitable one for the present purpose.

In the first of the experimental series, where equal numbers of hosts and parasites were used, six repetitions were made using individuals from a different stock each time. This gave a total of 150 females of *Microplectron* under observation. The results are shown in chart A of fig. 4.

In the second series, 65 females were faced with a definite choice between large and small hosts. Their behaviour is illustrated in fig. 4, chart B.

It will be seen that a certain degree of preference was exhibited for the larger cocoons in both cases. At the same time, a relatively large pro-

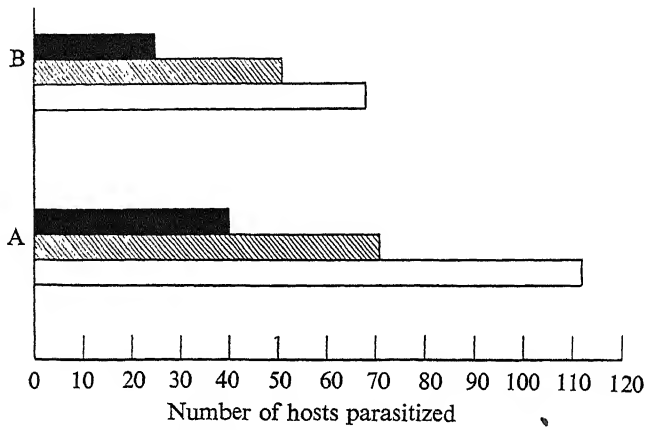

FIG. 4—Size of host and host selection. Series A contained equal numbers of parasite females and hosts with a total of 150 females. Series B gave each female a definite choice between a small and large host and a total of 65 females were tested. In both series the hosts were exposed to parasitism for 48 hours at 25° C. Bars shaded as in fig. 3.

portion of small cocoons was accepted even where an alternative choice of large ones was available. In both series, the number of small cocoons accepted amounted to approximately half that of the large cocoons. In view of this the results can hardly be taken as implying a discrimination under natural conditions, otherwise a much greater disparity between the two classes of host would be manifest in the laboratory experiments.

It seems reasonable to accept the view that, if otherwise suitable, a small cocoon is as readily acceptable when encountered by a parasite in the field as is a large, or normal-sized one. This is further supported by the fact that a small host is capable of supplying the food material necessary to bring to maturity the total progeny of a female *Microplectron*.

The point of size is mainly one of academic interest in the present instance. The small cocoons of *D. polytomum* actually constitute not more than 4 to 5% of the total population and the adults arising therefrom are usually males. From an economic point of view, therefore, it is of little importance if the small cocoons are ignored in favour of the larger ones.

2—*Discrimination Between Parasitized and Unparasitized Hosts*

Under the present heading it is proposed to deal with the acceptability, or otherwise, of hosts which have been used previously by females of the same species of parasite. The investigation was complicated by the occurrence of three factors, namely, (*a*) the presence of a cocoon enclosing the host completely obscures the latter from view and the degree of previous parasitism cannot be accurately determined before an experiment without undue disturbance of the host; (*b*) a single host is normally capable of supporting the entire progeny of more than one female if called upon to do so; (*c*) under laboratory conditions, two or more females commonly oviposit simultaneously in one and the same host.

In order to determine whether a host was parasitized before being used in the experiments, the cocoon was carefully slit along one side and the larva examined through the aperture. This was then closed and the cocoon glued down to the card with the slit underneath. In a preliminary trial the treatment in no way affected the acceptability of the host.

In nature a female parasite may encounter two classes of parasitized hosts. In the first of these the contained parasites will be still in the egg stage; in the second, hatching will have occurred and parasite larvae in various stages of development will be present. As it appeared quite possible that a difference such as this might be of importance in influencing the final acceptance of a host by the parasite, two series of experiments were carried out. In series A the parasitized hosts offered contained eggs of *Microplectron* only; in series B, parasite larvae were present in a nearly mature condition.

In both series, healthy and parasitized hosts were alternated on a card which was placed in a petri dish. Females were introduced so that each parasite was offered a definite choice between the two classes of hosts. The dishes were kept in an incubator at 25° C and 70% relative humidity, and an exposure of 48 hours was allowed. The whole of the cocoons were then examined for parasitism. In the previously parasitized hosts, the two distinct ovipositions could be separated with ease, as the prior

one had, by this time, given rise to very young larvae. In this manner the behaviour of 24 females in each series was under observation.

The results are illustrated in fig. 5. In series A the presence of parasite eggs on the host larva did not deter the females from further oviposition. The number of parasitized hosts chosen and accepted was equivalent to that of the healthy individuals used by the females. It is evident that no discrimination was exercised. In series B, on the other hand, there was a very marked disparity between the two classes of host material which clearly indicates the employment, on the part of the female, of some degree of deliberate choice.

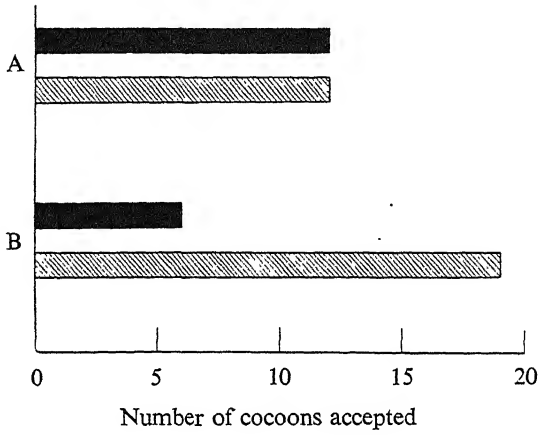

FIG. 5—Discrimination between parasitized and unparasitized hosts. In series A the parasitized hosts offered contained eggs of *Microplectron*. In series B the parasites within the hosts had developed to nearly mature larvae. Twenty-four females were tested in each series and a 48 hours' exposure was given at 25° C. Black bars = number of parasitized hosts accepted; shaded bars = number of healthy hosts accepted by the parasite.

The determination of the exact point at which this discrimination becomes operative is rendered almost impossible by the rapidity of parasite development during the initial larval stadia. Chance observations go to show that the presence of very young larvae, soon after hatching, do not affect the acceptability of the host and that it is only the later stages which produce rejection by a visiting female.

The cause of this distinction is difficult to ascertain by experimental methods. At the same time a reasonable explanation can be offered. Where parasite eggs are present on the host they are not readily perceptible to the female; there is no movement. The host larva, on the other hand, is paralysed but is still living and certain definite and recog-

nizable movements continue. It is quite possible that the parasite can detect these movements and will accept the host accordingly. By the time that the parasite progeny have developed to nearly mature larvae, the host is often dead, or very nearly so, and its movements are either very feeble or non-existent. The host may be rejected on this account alone. In addition, the presence of a large number of parasite larvae, all making characteristic movements, produces a type of vibration which is, possibly, very unlike that of the host and it is conceivable that the female parasite is able to recognize this difference. It should be remembered also that the chemical changes following the death of the host will produce an odour which differs from that of the healthy host. Whatever the explanation of this phenomenon may be, it is sufficient for our present purpose to know the actual course of events without seeking the underlying reasons for the behaviour of the female.

The findings in the present experiment have an important bearing upon any studies of the relationship between parasite and host. All population studies are hinged upon the way in which the parasite reacts towards the host under all possible conditions. In the present instance we are in a position to state definitely that the selection of the host from among a population is not entirely at random. It is true that, at any one time, a certain proportion of the population is dealt with in this manner, *i.e.*, the healthy hosts and those bearing parasite eggs. The portion bearing parasite larvae, however, is definitely rejected. Hence, this latter proportion must be ascertained and taken into consideration when making any computations based on relative host and parasite populations.

3—*Dead Host Larvae and Advanced Development of the Host*

In view of the foregoing results, it would be expected that the parasite can detect the presence of host larvae which are dead through disease or other cause, and of hosts which have reached a stage of development which is too far advanced to allow its progeny to feed, *i.e.*, when chitinization has commenced.

Although no definite experiments were arranged to determine this, a number of records were accumulated by dissection of such hosts when they were inadvertently mixed with healthy hosts during parasite rearing. These records show that the number of unsuitable hosts used for oviposition by the parasite was so small, in proportion to the total number examined, as to become insignificant. Among 319 hosts examined where the larva was dead, only 27 were accepted by the females concerned; while where the host had developed until the adult was almost formed

only 14 were accepted out of a total of 205 examined. There is, therefore, evidence for stating that the parasite is capable of making a discrimination in such cases.

VI—Discussion

The problem of host selection by *Microplectron fuscipennis* presents a relatively simple case and the various steps leading to the choice of a suitable medium for oviposition lend themselves to investigation by experimental methods. We have a parasite which is restricted to one genus of host insects, all the members of which possess similar characteristics and which is peculiar in other ways (*see* p. 265). The interpretation of the experimental results, therefore, cannot be applied to formulating sweeping generalizations for the majority of entomophagous parasites. The present discussion is mainly concerned with an endeavour to provide a satisfactory explanation of the way in which the female of *Microplectron* is led to find and accept the true host.

Discrimination between true and false hosts is accomplished with comparative ease under conditions nearly approaching the normal in the laboratory. It is certain that, in the field, very little time is wasted by the parasite on foreign objects. We are concerned, therefore, in studying the operation of those factors which lead to this discrimination. The initial discovery of a host depends mainly upon a chance encounter during the traverse of the particular area in which the parasite occurs. Chance rules until the parasite is within a certain distance from the host. In the laboratory this proved to be approximately 5 to 6 mm. At this point the host itself exerts an influence which procures an orientation of the female in its direction. This primary attraction has proved to be due to the odour of the host. Aided by the visibility of the object, the host is mounted and is subjected to a careful antennal examination, during which the character of shape and possibly that of texture, go to confirm the previous stimulus created by odour. The remaining external feature of size is most probably of no significance among individuals of the host population in its native environment. The host, as far as external characters are concerned, is acceptable to the parasite. It presents a combination of sense-pictures which is extremely unlikely to occur in any other object encountered by the searching female. At the same time, the chain of stimuli is still incomplete and the final acceptance depends upon the perception by the parasite of some further stimulus which must come, apparently, from the host itself, *i.e.*, from the living larva within the cocoon.

Absence of this stimulus may be due to the death of the larva, to advanced development, or to parasitism which has taken place previously. In all observed cases the rejection of the host offered has been found to depend upon one or another of these fundamental causes. The acceptance of a host, therefore, is conditional upon the host being alive at the time of examination. Previous parasitism may be divided into two classes according to its effect upon the phenomenon of host selection. Where it has not proceeded beyond the egg stage, or, at most, beyond the young larval stadia, the host is still living although paralysed. Under these circumstances, it is accepted by the parasite for oviposition. On the other hand, where the prior parasite progeny are in an advanced larval stage, the host is either dead or is in a very enfeebled condition. Such individuals are rejected as possible hosts.

As an explanation we are faced with two alternatives. In the first place, chemical changes after the death of the host larva may produce an odour which is repellent to the parasite. In this case the cause of rejection would operate at the very beginning of the cycle of events described above. Secondly, it is conceivable that larval movements on the part of the host are perceptible to the parasite. Paralysis of the host does not terminate all movements and, therefore, the presence of parasite eggs would not deter subsequent oviposition by other females. Where parasite larvae are well grown the host is often dead or feeble and movements have ceased in consequence. It is possible, also, that the vibrations set up by the parasite larva are readily distinguishable from those of the host. Difference in vibration would help to account for the avoidance as a host of those cocoons containing partially formed and chitinized adults.

It is clear, from the experimental evidence, that *Microplectron* is able to select its true host in the field with great accuracy and that, furthermore, it is able to exercise a degree of discrimination between the different conditions in which the individuals of the host population may occur. There is thus a definite proportion of this population which is not under the influence of random oviposition, as is the remainder. An attempt to summarize the behaviour of a female with respect to the selection of the host in nature is made in fig. 6 in a tabular form.

In view of the specialized nature of the parasite under review, it would be premature to attempt to offer any explanation of the behaviour of entomophagous parasites in general in the light of the facts elicited during the present study. We may, however, endeavour to compare the findings with the theories of insect behaviour which have been advanced.

At first sight it would appear that the experimental results are most

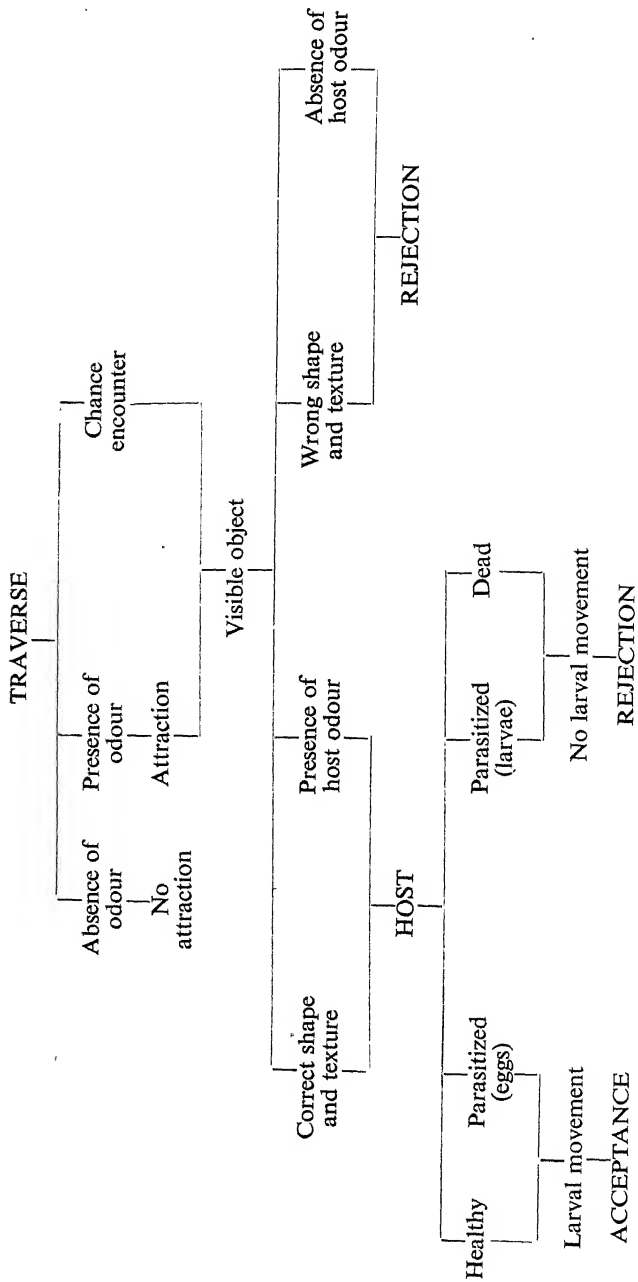

FIG. 6—Host selection by *Microplectron fuscipennis*. Tabular representation of the course of events in the field.

easily interpreted in terms of mechanical reaction to a stimulus and that the sequence of events is produced by a series of such responses to a pattern of stimuli. For the normal behaviour of the parasite this provides an apparently feasible explanation; but when a few of the instances of behaviour under abnormal circumstances are considered we must come to the conclusion that a wholly mechanistic view of host selection is untenable. Let us consider the case of a female which is confronted with a false host of the correct shape but possessing no other characteristic of the true host. We have already seen (p. 267) that shape is an important host character, but that shape alone will not induce a final acceptance of the object until after some time has elapsed. It *eventually does so*, however, in the absence of other characters (p. 276). An explanation of this phenomenon has been offered. If host selection is due to definite responses by the parasite to a pattern of stimuli emanating from the host (as would appear to be the case) and if these responses are purely mechanical, then the isolated character of shape should not produce acceptance of the object at all, no matter how long the female is confronted with the object. As, eventually, it does cause the female to accept the false host, the behaviour of the parasite cannot be entirely mechanical, but must be influenced by some disarrangement of its " mental " functioning. In other words, an " error of instinct " occurs, produced by the insistence upon a single trait of the normal host.

The fallibility of the parasite in this respect leads us to the conclusion that the underlying basis of behaviour during host selection is of a psychological nature. The experimental work has shown that, normally, the responses of the parasite to certain stimuli provided by the host are so definite that the initial reasons for the acceptance of the host by the parasite are susceptible to investigation by experimental methods. Inasmuch as this is possible, and in view of the conclusion to which we have been led, it seems that a pattern of sense pictures corresponding to certain traits of the normal and desirable host may be incorporated in the " mind " of the female parasite and that it is the correlation of these inherent abstractions with the actual physico-chemical attributes of the normal host that produces those apparently mechanical reactions which occur when parasite and host meet. This conception actually implies recognition of the host by the parasite. The healthy host, with its full complement of attributes, represents the normally desired object and the recognition of such, in its entirety, by the parasite awakens in the latter the desire to accept the object as a medium for oviposition. Where the host is lacking in one or more essential attributes it is still recognized by the parasite, but is recognized as an incomplete host. As only recog-

nition of the complete host is capable of awakening the desire to accept the object as a host, the incomplete host is rejected under normal conditions.

Further consideration of the experimental results supplies additional evidence of the psychological nature of the problem. It will be observed that we have been able to define the desirable host in terms of its physico-chemical properties and have been able to show that these play an important part in host selection. At the same time, the definition attained is not an exact one if we accept the standards of the physicist or mathematician. It is qualitative but not quantitative. In the latter case we should be required to give exact measurements of the factors involved which in turn should be applicable to every host accepted by the parasite. We are unable to do this, as will be clear from a moment's reflexion upon the nature of a normal host population, the individual members of which may exhibit a comparatively wide range of variation in their characters. Thus, if we take, as an example, the character of shape, we are confronted with the fact that while the *general* shape of a cocoon is fairly well defined, the individual cocoons of the particular species possess peculiarities of curvature which cannot be looked upon as a common feature.

The definition at which we have arrived is, in fact, a *general* and not an *exact* one. The female parasite encounters hosts which depart from the average conception in one direction or another and in accepting any one individual as a host it is exercising a latitude which can only occur if the process is fundamentally psychological.

It is obvious that we cannot choose any particular characteristic of the host and point to it as the main criterion used by the parasite in its selection. We might say, for example, that a particular parasite selects its host on account of size. If unaccompanied by a definite dimensional qualification, "size" becomes an undefined abstraction. The statement that "size" is meant to indicate a certain length, breadth, or area improves the situation slightly but does not remove the fundamental difficulty, namely, that "size" is not a concrete object and it is clearly a concrete object, alone, which can awaken the desire to oviposit.

If size, however, is regarded as *one* of the essential component attributes of the host it immediately assumes some significance, as it is now part of a concrete object. It is a mistake, therefore, to refer to size as the main attribute of the host, inasmuch as the presence of the remaining characteristics is equally essential to the composition of the complete host. Without these other attributes the host would cease to exist as an object capable of being recognized by the parasite. The same argument can be

applied to each of the other characteristics embodied in a normal host, when considered separately. We are forced, therefore, to look upon the individual attributes of the host as equal partners in the whole economy of the host-parasite relationship.

The above remarks have been based upon the study of a monophagous parasite, but it will be realized that they can be applied also to the case presented by polyphagous parasites. With the latter the problem offered by the different species of hosts used by the parasite is, in reality, a problem of the same order as that of the different individuals taken by a monophagous parasite. The only real difference in the content of the definition it is possible to give in each case is one of degree. With a monophagous parasite we are able to make a statement which is more definite and less broad than is possible with a polyphagous species.

There is a very real need for development and improvement in methods for the experimental study of insect behaviour. That the subject is an important one from the standpoint of practical biological control serves to emphasize this need. We actually know very little of the causes of insect behaviour; our present stand is based, for the most part, upon surmise. This is due, no doubt, to the peculiar difficulties of the problem and to the absence of a suitable technique.

A few suggestions are contained in the present paper, which may prove useful in the future development of a technique. An emphasis has been laid upon the factor of time in all host selection studies. Isolation of false hosts from the influence of true hosts has been advocated, and it has been shown how the former can be used to study the single characters of the true host. It has been considered, throughout, that all possible factors leading to abnormal conditions should be eliminated at the commencement of an experiment and some of these factors are discussed. Some abnormalities are bound to remain, however, and these should be allowed for in the interpretation of the results. The latter must be drawn up with very real appreciation of field conditions. This has not always been done. A conclusion which is unreasonable when viewed from the angle of a practical field entomologist, is worse than useless.

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VII—Summary

A brief review of the general situation regarding host selection among entomophagous parasites is presented and the present extent of our knowledge discussed.

The nature of the present problem is described with reference to the peculiarities of *Microplectron* in relation to its host material.

The various factors influencing the selection of the host by the parasite are examined experimentally. It was found that *Microplectron* was able to discriminate with ease between true and false hosts even where the latter resemble the normal host in everything except the presence of the living larva within the cocoon. Evidence seems to suggest that acceptance of a host depends, to a large extent, upon the presence of larval movement. A new technique was developed, during these studies, which removed a number of objections present in previous methods.

The selection within the host species as represented by choice between parasitized and healthy individuals and also between the latter and unsuitable hosts was investigated. A high degree of discrimination existed between healthy material and hosts containing parasite larvae which were well grown. The presence of parasite eggs, on the other hand, did not deter females from ovipositing in the host. The reason for this difference is thought to be larval movement, which is present in the latter and absent in the former case.

There is thus a definite proportion of every host population which is not subject to random oviposition.

The behaviour of the parasite is discussed and a reasonable explanation is offered.

It is shown that a wholly mechanistic view of host selection is untenable and that the underlying basis of behaviour is of a psychological nature.

The necessity for developing and improving our existing technique for use in the investigation of problems of insect behaviour is emphasized and suggestions from the present study are put forward.

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A New Group of Filterable Organisms

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[PLATE 12]

A group of organisms of small size has been discovered which can be cultivated in indefinite series. The organisms form a well-defined group and have both relatively large and small forms. The smaller forms appear to be as small as vaccinia virus and from them the larger forms readily develop.

OCCURRENCE

The organisms have been obtained from all samples of raw sewage so far examined from four London districts during the summer. They have not been detected in London tap-water, or faecal material from man, pig, rabbit, or rat.

ISOLATION

The organisms are readily obtained by successive filtration of raw sewage through " Gradocol " membrane filters (Elford, 1931) of diminishing porosity. Samples of about 15 to 20 cc of sewage are mixed with about 10 cc of nutrient broth and shaken thoroughly to break up clumps of bacteria and protozoa. The mixture is first clarified by centrifugation or filtration through paper pulp to remove any large aggregates, and different samples are filtered through membranes with average pore sizes of 1·0 μ, 0·8 μ, and 0·6 μ. The membrane filtrates are sown in quantities of 0·5 to 1·0 cc into Fildes's broth, or onto Fildes's agar slopes, and incubated at 30° C for some days. The first sign of growth in the broth tubes is an alteration in the colour of the medium from a warm brown tint to a dirty yellow; this is soon followed by a hazy appearance which gradually develops into a definite but not always pronounced turbidity. With the solid medium it is advisable to flood the fluid at the bottom of the agar slope half-way up the exposed surface after two or three days and incubate again for some days longer. Growth is indicated by the development of a ground glass appearance on the lower part of

the slope with an obvious high water mark showing the limit of the flooded area. Subculture is readily effected from solid or liquid cultures by transference to fresh tubes of medium.

On one occasion one of these organisms was recovered on Fildes plates by the plating methods used in routine bacteriology. This indicates that the organism must be present in sewage at times, in fairly large numbers. In filtrates the numbers are naturally reduced, but 0·5 cc samples of 0·8 μ membrane filtrates usually give positive results.

In this study all strains were purified by plating high dilutions of the cultures and picking single colonies under a low power binocular microscope and working with the cultures resulting therefrom.

In many early experiments other types of medium were inoculated and other organisms were recovered from the coarser filtrates such as spirochaetes, and an organism apparently identical with *Spirillum parvum* of von Esmarch (1902). This organism grew freely on highly dilute nutrient broth but very badly, or not at all, on Fildes's or other rich medium. *Spirillum parvum* was studied to some extent as it is of historical interest and is readily filterable through filters of the Berkefeld type. This organism was also recovered from London tap-water. As a rule all obvious bacteria were discarded and attention confined to organisms suspected to be of virus size.

Cultivation

The most generally suitable medium so far discovered for these new organisms has proved to be Hartley's digest broth made from horse meat, set to a reaction of about p_H 8·0 and enriched with a peptic digest of red cells as described by Fildes (1920) for haemophilic bacteria. Media with a neutral reaction give very inferior results, and those with a reaction more acid than p_H 7·0 give no sign of growth. Alkaline serum broth is also suitable for the majority of these organisms but proved useless for one strain. Alkaline broth without serum enrichment gives irregular results. Serum agar slopes, and blood agar slopes, give a delicate growth but are inferior to Fildes's agar. Blood broth gives a good growth but is inconvenient. Growth in liquid medium is accompanied by the development of a fine haze which progresses to a slight turbidity; this reaches a maximum and thereafter declines leaving a faintly hazy medium with a small granular deposit. There is no lysis of red cells in blood broth nor alteration in the colour of the haemoglobin in media containing blood. In serum broth the growth is more granular and there is usually an obvious if small deposit at the bottom of the tube.

This is due to the organisms growing in clumps of hundreds or more. Dilution of a good medium with water rapidly converts it into an inferior one, and in view of the natural habitat of the organisms this is rather surprising. On the whole growth is slightly better under aerobic conditions, but anaerobic conditions are quite suitable for all strains.

The optimum temperature for recently isolated organisms appears to be about 30° C, though growth proceeds quite well, if more slowly, at room temperature (22° C), and satisfactory cultures are usually obtainable at 37° C when large inocula are used. At the highest temperature some irregular results were experienced and colony formation was less perfect.

The organisms are readily killed by heat. Heating to 45° C for 15 minutes destroyed the majority of them and none withstood 55° C for 5 minutes.

Growth on solid medium can be very deceptive as it is often fine and delicate. When the whole of the surface is flooded with inoculum the only change to be observed is a faint ground glass appearance which can easily pass undetected. It is always advisable to leave a fair proportion of the surface uninoculated so that a contrast between the normal surface of the medium and the growth-covered area can be obtained. When very dilute inocula are employed on a large solid surface and incubation is prolonged for five or six days giant colonies up to 0·35 mm may be obtained on suitable medium. The colonies when fully developed are umbonate; the centre is rough and usually of a pale brown colour; the periphery is flatter and smoother but may show irregular radial markings. Quite young colonies are usually lenticular, colourless, and quite smooth.

It is possible to count the number of viable organisms in a fluid culture by plating a known volume of an appropriate dilution onto solid medium, incubating for 4 or 5 days, and counting the separate colonies under a low power binocular microscope. The procedure is tedious but gives useful information. Growth curves may be drawn, and it is found that a medium which looks clear may contain 40,000,000 organisms per cc, and the most turbid culture may contain 3 to 10 thousand million organisms per cc. In old cultures the population diminishes and this is accompanied by considerable clearing of the medium. From the counts in developing cultures the generation time during the logarithmic phase of growth was calculated for strain A on the assumption that simple division occurred. This was found to be about 1·6 hours at 30° C, and 1·1 hours at 37° C after the strain had been cultivated under artificial conditions for some months.

Morphology

Dark ground illumination of the living unstained organisms gives the most reliable information regarding their structure. A thin film of a young culture of any of these organisms grown on Fildes's broth, examined by dark ground illumination with a 1/12-inch objective, funnel stop and a 12 X or 15 X ocular shows large numbers of bright rings and minute particles. The largest elements appear to be spherical in some instances and discoid in others. They vary in size from large forms which look like small cocci (about 0·5 μ in diameter) down to small dots which are only imperfectly resolved by the optical equipment specified. The rings are usually single but may be paired. Occasionally the ring appears to show a granule or have a local thickening at the periphery, in other instances a short filament may be attached to the margin of the ring. In older cultures local thickenings giving a signet-ring appearance are more common and large bloated forms may also be seen which may have one or more interior granules.

In cultures grown on medium containing serum the rings are clumped together in aggregates of a hundred or more and there are relatively few free forms. Study of a number of preparations from such cultures shows that the rings are connected together by very fine filaments. At the margins of the aggregates rings may, from time to time, be seen attached to the main mass by a thin filament giving a drum-stick appearance. When the clumps are loose the whole mass may flicker as the spheres and filaments sway about under molecular bombardment.*

Stained preparations are not quite so satisfactory for we are convinced that the organism is readily distorted. Films are best prepared from cultures on solid or liquid media on cover glasses. The wet films are fixed by floating on saturated mercuric chloride to which acetic acid up to 5% has been added. They are then dealt with as wet films and never allowed to dry during staining or mounting. Giemsa's stain, carbol fuchsin, and Heidenhain's iron-haematoxylin have been found to be the most suitable stains. Successful preparations show rather more diversity of form and size than is observable under dark ground illumination. Large coccoid bodies are prominent but many small forms can be seen down to granules which are so small that one is in doubt as to whether they are organisms or particles from the medium (*see* fig. 1, Plate 12). Some of the larger forms stain more deeply at the periphery, which is

* It is hoped that J. E. Barnard will, in the future, give an account of the microscopic appearances of these small organisms as revealed by more refined optical methods.

what is to be expected if the form of the stained body is a biconcave disc; others stain more uniformly, which is the expected picture from a spherical body. The smallest bodies appear uniformly stained, but their proper resolution is impossible. Here and there thin rod-like structures are to be seen; these may be distorted organisms or, as we consider equally possible, discoid bodies viewed on edge. In preparations from older cultures forms with irregular thickenings at the margin can be detected.

It will be observed that these organisms show structures which recall a number of those which are met with at certain stages in cultures of the organism of bovine pleuropneumonia.

We have not been able to determine in what way the organisms multiply. It is certain that large forms may develop from the smaller, but whether the organism multiplies by separating off small particles or not is undecided. We never see septa in the larger ring forms which are so striking in actively growing cultures of staphylococci and we regard it as improbable that multiplication occurs by simple fission of the larger forms. We believe that there is some more complex method of development. Attempts were made to observe directly the division process under the microscope in a hot room at 37° C but these were not successful. It was shown that light rays did not interfere with the multiplication of the organism, but when it was discovered that the generation time was more than one hour assuming a simple method of division, this line of study was abandoned.

Biochemical Activities

It was hoped that it would be a simple matter to determine the nature of the foodstuffs required for the growth of these organisms but the results have been, so far, very disappointing. Whenever growth occurs acid is developed and when the reaction reaches p_H 7·0 or thereabouts growth ceases. The change in reaction has been followed by colour changes with indicators and more accurately with the glass electrode. None of the sugars usually employed in typing bacteria favoured the growth of these organisms and there was no sign of fermentation. This result may be associated with the failure to grow whenever the reaction becomes faintly acid. However that may be, media which gave typical fermentation results with the organism of bovine pleuropneumonia gave negative results with the new organisms, though it was demonstrated that active growth had occurred. Ammonia is not produced from urea. Sodium tellurite is slowly reduced but nitrates are not reduced to nitrites. The haematin of Fildes's medium is partly converted under anaerobic conditions into haemochromogen, and later is altered in some way, being

converted into a yellow pigment the nature of which is unknown. The addition of sodium salts of a number of organic acids to the standard rich culture medium did not produce increase of growth or the development of an alkaline reaction. In brief the metabolic activities of these small organisms, even though the population may be large, are not great enough to be followed by rough and ready methods, and greater refinement will be required.

The precise role of the Fildes enrichment is obscure. The addition of separate X and V factors (haematin and yeast extract) did not give so good results as a peptic digest of red cells. Moreover there is some constituent in the Hartley broth which is essential for copious growth. None of the commercial peptones can be substituted for digest broth if good growth is desired. Casein digests we found to be useless.

Multiplicity of Strains

We have isolated three strains which show small but definite differences in cultural behaviour corresponding to well-defined serological differences, but morphologically the three organisms appear to be identical. Strain A was isolated most frequently and was encountered in sewage from Finchley, Hendon, and Croydon. It grows more freely than the others and produces a more pronounced turbidity in liquid cultures. Digested blood is not indispensable for its development though this addition is certainly advantageous. Serum broth is quite suitable for maintaining indefinite subculture in series and even ordinary nutrient broth made alkaline will permit of fairly satisfactory growth. Strain B, obtained from Hornsey sewage, is very similar in growth requirements to A but shows a great tendency to grow in masses at the bottom of a culture tube (particularly if a little serum is present), and the supernatant fluid is often quite clear. Strain C does not grow at all readily and requires digested blood for its development. It never produces a pronounced turbidity, and gives very small colonies on the surface of solid media. Strains A and B give colonies up to 0·35 mm, and strain C up to 0·17 mm. The colonies of strain C are not umbonated but rough and irregular on the surface. Strain C when originally isolated showed two different sizes of colony and these were picked off and grown separately. For a time the larger colony bred true and gave large colonies and the small always gave small, but on prolonged subculture mixed sizes of colonies developed in both instances. At present we regard the three strains, A, B, and C, as variants of a single group on account of their morphological and cultural similarities.

Serological Reactions

Two rabbits were immunized by a series of intravenous injections of suspensions of organisms of strains A and C respectively. The organisms were grown in Fildes's broth, centrifuged down and suspended in a small volume of saline prior to injection. The injections caused no bodily disturbance in the rabbits. After five injections at four-day intervals, samples of serum were collected and found to agglutinate the corresponding organisms very well. Tests with this serum are shown in Table I. It will be observed that antiserum to strain A agglutinates and precipitates strains from Finchley (i) and (ii), Croydon, and Hendon; has only a slight action on a strain from Hornsey (strain B) and no action at all on strains C (from Finchley sewage) or pleuropneumonia. Conversely antiserum for strain C has no effect on the A and B type organisms, but shows a striking action on strain C. There can be no doubt that strains A and C are quite distinct antigenically, and that strain B is more closely related to A than to C. None of the strains shows any relationship to pleuropneumonia and corresponding with this, antiserum to the latter organism is without action on any of the strains of the new organism.

After six weeks' rest the rabbits were given four further injections and the final serum harvested. The only difference observable in the serum after further immunization was that the antiserum to strain A now produced much more striking agglutination of strain B. Strain C remained unaffected, and strain C antiserum remained without action on both A and B. Normal serum from man, rabbit, sheep, pig, goat, ox, or horse did not agglutinate the organisms.

Ultra-filtrates of old cultures of these organisms also give small precipitates with the corresponding antisera showing that a precipitable substance is formed or liberated in the culture medium.

Filterability of the Organism and Size of the Smallest Units

Preliminary filtration experiments with Fildes's broth cultures suggested that the size of the smallest phase of the organism was probably about 0·2 μ, since membranes of average pore diameter (A.P.D.) 0·42 μ gave positive filtrates while 0·25 μ membranes yielded sterile filtrates. In view of the variable size of the elements seen under the dark ground microscope it was decided to establish the complete curve showing the filterability of the organism through membranes of porosities ranging

TABLE I

Organism		A^r_A antiserum					C antiserum				
		½	⅛	1/32	1/128	1/572	½	⅛	1/32	1/128	1/572
A	Finchley (i)	++	++	++	++	0	0	0	0	0	0
	Finchley (ii)	++	++	++	0	0	0	0	0	0	0
	Croydon	++	++	++	0	0	0	0	0	0	0
	Hendon	++	++	++	+	0	0	0	0	0	0
B	Hornsey	+	0	0	0	0	0	0	0	0	0
C	Small colony	0	0	0	0	0	++	++	++	+	0
	Large colony	0	0	0	0	0	++	++	++	+	0
Pleuropneumonia		0	0	0	0	0	0	0	0	0	0

Shows the degree of agglutination of the organism specified by the two specific antisera at the final dilutions indicated. Equal volumes of cultures and dilution of antisera mixed and incubated at 39° C for four hours.

++ = complete agglutination with precipitation.
\+ = agglutination without precipitation.
0 = no sign of agglutination.

from 1·0 μ down to the filtration end-point. A Fildes serum broth culture of strain A, grown for two days at 30° C, was diluted with an equal volume of Fildes's broth and then filtered through a membrane of A.P.D. 1·4 μ. This provided a filtrate free of any aggregates, and the concentration of the organism, found by making Fildes's broth cultures of serial tenfold dilutions, was 10^9/cc. Measured 7 cc amounts of this filtrate were filtered under 76 cm mercury pressure through selected membranes ranging in porosities from 1·0 μ to 0·22 μ. The results are contained in fig. 2. It will be noticed that upwards of 90% of the organisms may be retained by relatively porous membranes of A.P.D. 0·7–1·0 μ, yet it is not until porosities less than 0·25 μ are reached that complete retention is assured. The least porous membrane to yield a

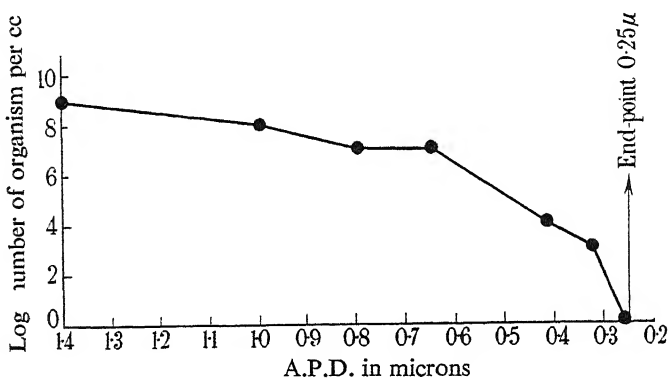

FIG. 2—Filtration end-point for strain A grown in Fildes's serum broth medium at 30° C.

positive filtrate has been of A.P.D. 0·26 μ. The filtration end-point 0·25 μ coincides with that previously found for vaccinia virus (Elford and Andrewes, 1932). The curve of fig. 2 is interpreted as indicating the smallest phase of the organism to be 0·125–0·175 μ in particle diameter, but that there are in addition larger forms ranging up to 0·5 μ in diameter.

In its general filtration behaviour the organism resembles bovine pleuropneumonia and agalactia, and contrasts with viruses and bacteriophages, which, individually, have been found to be relatively uniform in particle size, as evidenced by the fact that no appreciable drop in filtrate concentration, as compared with the original, is detected until the porosity of the membrane used is about twice the true end-point value.

The filterabilities of all the strains studied have paralleled closely that of the particular strain described above in fig. 2. Strain B (Hornsey)

presented difficulties owing to the pronounced tendency to spontaneous aggregation, which made it appear to filter rather less readily.

It is significant that cultures prepared from filtrates yielded by 0·28 μ membranes (this grade completely retains particles greater than 0·2 μ in diameter) behaved in subsequent filtration experiments exactly like the parent culture. They also presented a similar picture when examined under the dark field microscope. It is evident therefore that the smallest phase of the organism can initiate its development and growth process.

Animal Experiments

The organisms appear to be simple saprophytes. Two strains, A and C, were fed to rats, injected intravenously into rabbits, given by inhalation, by subcutaneous and intraperitoneal injection to mice and no ill-effects followed. The two strains of organism were readily recovered from two mice which were killed three hours after intraperitoneal inoculation; at this time there was a good but not striking polymorphonuclear cell exudate in the peritoneal cavity. At twenty-four hours cultures from the peritoneum of two other mice similarly inoculated proved to be sterile and mononuclear cells were now dominant in the cell exudate. Four further mice remained normal for over a week and no abnormality could be detected post-mortem.

Discussion

The chief interest in this group of micro-organisms seems to us to centre round the small forms which are to be found in all cultures. The existence of these suggests that the organisms may form another connecting link between the larger bacteria and the pathogenic viruses; and certain appearances we have described indicate that these organisms may be related in some way to the organism causing bovine pleuropneumonia. Other workers have described small organisms which pass bacterial filters but so far as we are aware no one has described this group.

We do not consider that this group is related in any way to the " filterable forms " of the pathogenic bacteria which have been described by many, *e.g.*, Hadley, Delves, and Klimek (1931), or Kendall (1931 and 1932). The existence of these filterable or " virus " forms is still contested, but in any case they arise under ill-defined conditions or as the result of special treatment (Hadley *et al.*), or on a particular kind of medium (Kendall). The " virus " forms are said to multiply for a time, as such, but may, under appropriate conditions, revert to the larger form

which, in turn, multiplies as such. During eight months' study of several strains of the new organisms we have invariably found the small and the large form side by side and we are of the opinion that the small form will, some day, prove to be merely a regular stage in the development of the large.

Oerskov (1931) described what he regarded as minute organisms growing, on saccharose agar plates, symbiotically with a special bacillus which he grew out of milk. Dienes (1933–34) also has described small colonies around colonies of certain strains of *B. subtilis* on, or in, saccharose agar medium. These he believes to be formed by a small symbiotic organism. Neither of these workers succeeded in separating the small organism and obtaining growth in serial subculture. Klieneberger (1935) has recently described a small organism growing in association with *Streptobacillus moniliformis*, Levaditi, which is regarded by her as showing certain resemblances to the organism causing bovine pleuropneumonia. In an addendum to her paper she states that she has succeeded in growing the small organism separately in pure culture. The relationship of these symbiotic organisms to ours is quite uncertain. From the descriptions there would appear to be great morphological differences and close comparison of the various organisms by one observer would seem to be required.

Barnard (1935) gave an account of the microscopic appearances of certain "saprophytic viruses" which he discovered in culture media. Unfortunately he was not able to secure indefinite serial subculture of the organisms and direct comparison of cultures is now impossible.

We thus do not know how our organisms should be classified but we feel it is highly desirable that the method of multiplication should be established for this would probably determine its classification and moreover might have important bearings on kindred studies with pathogenic viruses. Our study has shown again that organisms at least as small as the vaccinia virus, can lead an independent existence and that small size alone cannot account for our failure to cultivate any of the viruses on artificial media. Moreover it seems desirable that the nutritional requirements of these small organisms should be accurately defined, for it is possible that exact knowledge of this sort might enable us to cultivate some of the pathogenic viruses apart from living cells.

Summary

A group of filterable, saprophytic organisms has been discovered and described. These organisms appear to be closely related to each other

FIG. 1—Impression preparation from Fildes's agar plate culture. Stained Heidenhain's iron haematoxylin. × 1250.

(*Facing p.* 302)

and in the normal course of their development have small forms about the size of vaccinia virus. They may be cultivated on artificial media in indefinite series and may possibly prove to be a link between the larger bacteria and the pathogenic viruses.

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The Innervation and Reactions to Drugs of the Viscera of Teleostean Fish

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Introduction

During recent years several workers have studied the innervation and responses to drugs of the viscera of Selachians (Lutz, 1931; Nicholls, 1933 and 1934), but much less attention has been devoted to the teleostean fish. Müller and Liljestrand (1918), in the course of their work on Selachians, investigated briefly the movements of the viscera of various freshwater Teleosts, finding that stimulation both of vagus and splanchnic nerves caused movements of the stomach. Dreyer (1928) showed that the stomach muscle of various marine fish contracts upon addition of pilocarpine, but relaxes with adrenaline.*

* Since this paper was communicated to the Society my attention has been called to the paper of Méhes and Wolsky (1932) on the musculature of the gut of the Tench (*Tinca*). I have not been able to consult the original of this paper, but according to

Investigation of the innervation of the viscera of these fish has probably been delayed by the awkward anatomical conditions in the majority of Teleosts. Not only are the organs closely packed in a narrow space, but usually the nerves, and particularly the sympathetic chains, lie in a deep groove below the vertebral column and are covered by dense layers of pigment. For these reasons no attempt was made during the present work to use ordinary fish, but advantage was taken of the more favourable conditions found in two angler fish, *Lophius piscatorius* and *Uranoscopus scaber*. These two animals are not very closely related and they "angle" in quite different ways, yet both possess certain characteristics which make them very convenient laboratory animals. They are flattened dorso-ventrally, giving easy access to the viscera, they are almost free from internal pigmentation, and, most important of all, the branches of the vagus and sympathetic system can very easily be discovered and stimulated. The anatomy of the autonomic nervous system of *Uranoscopus* has already been described in some detail (Young, 1931) and that of *Lophius* is essentially similar.

Uranoscopus can be obtained at the Zoological Station at Naples, and the investigations were made there during the summer of 1931; I have to thank Professor R. Dohrn and his staff for much assistance given at that time. *Lophius* was studied at Plymouth during the summers of 1933 and 1934. The fish are common, but can only be kept alive for at most a few days in the tanks. My warmest thanks are due to Dr. Allen and the staff of the Marine Biological Association, especially for the trouble which they took to obtain *Lophius* alive. Professor E. S. Goodrich and Dr. J. C. Eccles have kindly criticized this paper, and Sir Henry Dale gave valuable assistance during preparation of the manuscript.

Anatomy and Innervation of the Gut

In *Uranoscopus* the food consists of small fishes and Crustacea, which are swallowed whole and received through a short oesophagus into a large and very muscular stomach. The cardia and pylorus lie close together anteriorly, the stomach thus forming a sort of bag in which the prey is collected. Around the first part of the duodenum are set the pyloric caeca, characteristic of Teleosts; in *Uranoscopus* they are well developed

the summary in 'Ber. wiss. Biol.,' vol. 26, p. 640 (1933), the authors found layers of striped as well as smooth muscle, both layers being innervated by the vagus but not by sympathetic nerves. "Parasympathetic" drugs stimulated the smooth muscles, but "sympathetic" drugs were without effect. Atropine inhibits the effect of the vagus on the smooth muscle. The absence of any sympathetic innervation would be a surprising difference between the tench and the fishes here discussed.

and comprise about 20 hollow, thick-walled tubes. There is a very large gall bladder and the bile duct has very thick muscular walls. The intestine is short, consisting only of a single loop and a straight limb running back to the rectum.

The gut of *Lophius* is essentially similar to that of *Uranoscopus*. The stomach is an enormous bag with very thick muscles, mainly longitudinal.

The nerves to the gut are similar in the two fish and include only the two vagi and splanchnic nerves, fig. 1. The former are unequally developed; that on the left being smaller and limited in distribution to its own side of the stomach, whereas the larger right vagus branches extensively on that side of the stomach and may perhaps also send branches on to the intestine, though none has actually been traced, either anatomically or physiologically during the present work.

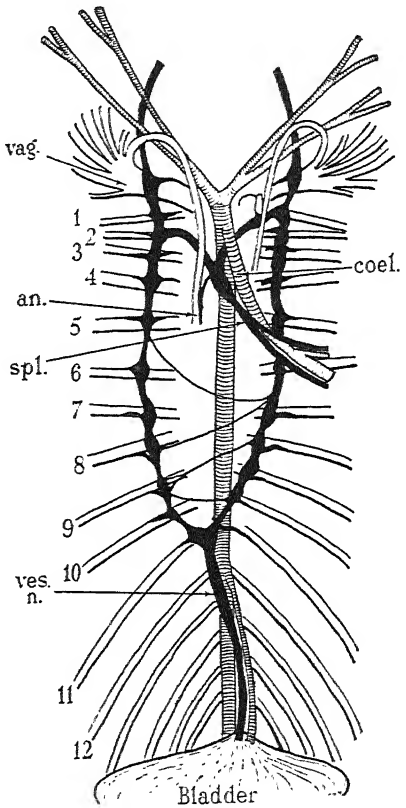

FIG. 1—Diagram of the abdominal sympathetic system of *Lophius piscatorius*, seen from below. *an.*, anastomosis between splanchnic nerve and R. vagus; *coel.*, coeliac artery; *spl.*, splanchnic nerve; *vag.*, R. vagus nerve; *ves. n.*, vesicular nerve; 1–12, spinal nerves.

The splanchnic nerves are also unequally developed, that on the right being the larger. Each springs from the first spinal sympathetic ganglion, and the two usually join in a large splanchnic ganglion which lies on the right side close to the origin of the coeliacomesenteric artery. From this ganglion nerves can be traced along all the major branches of the arterial system to the stomach, intestine, gall-bladder, spleen, etc. There is usually also a large branch connecting the right vagus with the splanchnic ganglion. Careful search has been made for nerves from the hinder sympathetic ganglia to the intestine or rectum, but such have never been found in either animal. Neither have movements of these viscera ever been observed following stimulation of the sympathetic ganglia in this region. However, there are so many small branches running in the mesenteries of the bladder and ovaries (*see below*)

that it is difficult to be *certain* that none runs to the intestine; the point requires further careful experimental investigation.

Functions of the Nerves to the Gut

(*a*) *Uranoscopus*—In order to obtain a preparation for the study of the effects of the nerves of the stomach the animal was pithed, the abdomen opened, and a ligature tied round the oesophagus and attached to a nail in front of the head. By attaching a hook to the posterior end of the stomach and linking this up with a lever a record of the movements of the longitudinal muscles of the stomach could be obtained. The disadvantages of this method compared with manometer or balloon readings are obvious, but since the most active muscles are longitudinal it is possible to obtain some idea of their behaviour. During the experiment the viscera were moistened with the physiological solution suitable for marine Teleosts which has been described elsewhere (Young, 1933, *a*). The solution used for *Uranoscopus* was exactly that described in the paper and contained 1·35% NaCl. At Plymouth determinations of the freezing point of the oxalated blood of *Lophius* gave −0·703° C and −0·701° C for two animals, and it was accordingly calculated that the appropriate saline medium should contain 1·22% NaCl. The solution used for *Lophius* at Plymouth therefore contained the following quantities of M/2 salts:

NaCl	410 ml
KCl	16 ml
$CaCl_2$	4·5 ml
$MgCl_2$	7·3 ml
$NaHCO_3$	4·5 ml
KH_2PO_4	1·0 ml
H_2O	To 1 litre.

Under these conditions the stomach either remained at rest or else showed slight contractions at irregular intervals. Faradic stimulation of either vagus nerve using "unipolar" or "bipolar" silver electrodes always caused very marked contraction. The effect was similar whether the vagus was stimulated inside the skull or where it runs to the stomach.

Stimulation of the sympathetic chain at the level of the 1st to 4th spinal nerves, or of the splanchnic nerves, usually caused a slight contraction of the wall of the stomach, but this was very delayed and was always very much less than that which follows stimulation of the vagus.

The intestine, lying free *in situ* with all the nerves intact, usually showed adanal peristalsis near the pylorus but elsewhere remained quiet. Stimu-

lation of the vagus was never followed by any visible movements in the intestine, but stimulation of the anterior sympathetic ganglia, or of the splanchnic nerves, was followed by waving movements of the pyloric caeca and by the initiation of adanal peristalsis in the whole intestine and rectum. As in Selachians, therefore, the sympathetic of *Uranoscopus* supplies motor fibres to the gut posterior to the pylorus; but in *Uranoscopus* the fibres all run out anteriorly in the single pair of splanchnic nerves instead of segmentally as in *Scyllium*.

Stimulation of the anal sphincter nerves (Young, 1931) caused movements of the spines behind the anus, but no detectable movements of the rectum, bladder or ovaries. In order to determine the effects of these

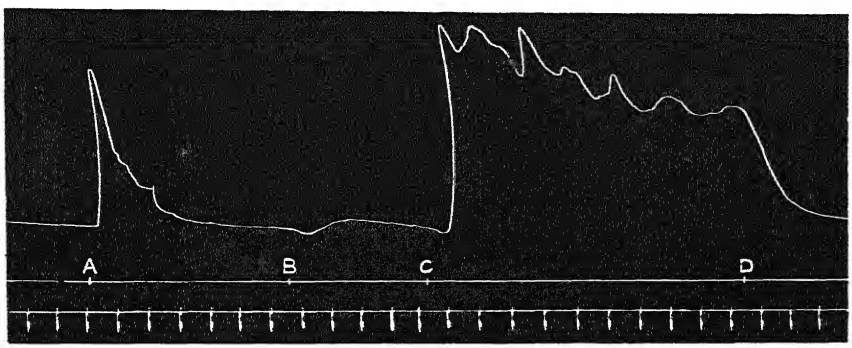

FIG. 2—*Lophius*, stomach, longitudinal muscle. At A, faradic stimulation of vagus nerve, secondary coil 8 cm. Add drugs to make, at B acetylcholine 1/1,000,000; at C acetylcholine 1/100,000; at D adrenaline 1/100,000. The time in all of the figures is marked in minutes.

nerves on the sphincters it would be necessary to isolate the latter, a difficult operation in *Uranoscopus*, but perhaps possible in *Lophius*.

(*b*) *Lophius*—Essentially similar results were obtained by stimulation of the nerves to the gut of *Lophius*. Stimulation of either vagus was followed by powerful contraction of the muscles of the stomach. The walls are so thick that it is very easy to dissect away a piece of the longitudinal muscle with the vagus nerve attached, and good nerve-muscle preparations can be obtained in this way (fig. 2). Stimulation was by means of bipolar metallic electrodes, over which the nerve was stretched in a vulcanite holder. Since the electrodes dipped below the surface of the liquid in the bath rather strong stimuli were needed. The possibility of escape of current was controlled at the end of each experiment by pinching the nerve.

Stimulation of the splanchnic nerves was not followed by such definite

movements as in *Uranoscopus*, but slight movements were sometimes seen in the wall of the stomach near the pylorus and also regularly in the pyloric caeca and duodenum.

Since the above methods of investigation are somewhat crude, it is as yet only possible to give general statements as to the more obvious functions of the nerves. All the evidence agrees that in these fish the vagi contain motor fibres for the stomach, and the splanchnic nerves motor fibres to the entire duodenum and intestine. It remains to be discovered by more accurate work whether the vagus affects the intestine, or the

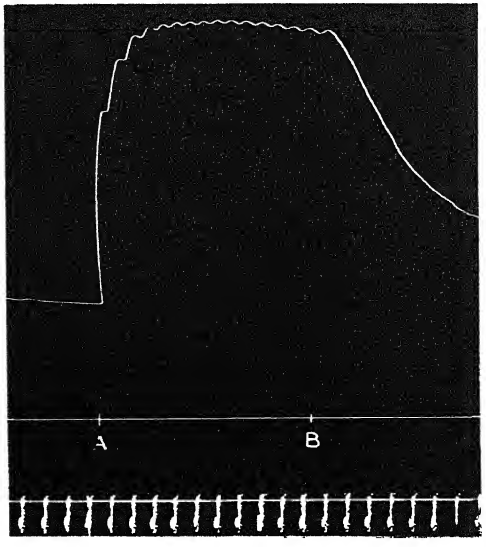

FIG. 3—*Lophius*, stomach, circular muscle. Add drugs to make, at A acetylcholine 1/500,000; at B adrenaline 1/500,000.

splanchnic the stomach, and whether such reversals as those detected in mammals by Brown and Garry (1932) with different frequencies of stimulation, and in differing states of distension occur also in fish.

EFFECTS OF DRUGS ON THE GUT

Pieces of the gut musculature were removed and placed in saline mixture in a wide tube, and attached to a recording lever in the usual manner. Oxygen or air was bubbled slowly through the liquid in the bath.

A piece of the longitudinal stomach muscle of *Uranoscopus* isolated in this way either showed slight and irregular contractions or remained at rest. The addition of adrenaline hydrochloride* to make 1/1,000,000

* Solutions made up freshly before use by neutralization of the base.

was followed by a very marked fall in the tonus of the muscle and if there had been spontaneous contractions these were completely inhibited (six experiments). Addition of acetylcholine bromide (B.D.H.) to make 1/1,000,000 was followed by a rise of tonus and the initiation of contractions if not already present. Acetylcholine was found capable of causing a rise of tonus after inhibition by adrenaline and there is a quantitative balance between the two. For example, in one experiment acetylcholine 1/1,000,000 prevented the loss of tonus which was resulting from the action of 1/1,000,000 adrenaline, but rhythmic contractions only began again after the addition of acetylcholine to make 1/250,000.

The behaviour of the stomach muscle of *Lophius* was similar, with the exception that it rarely showed spontaneous contractions. Acetylcholine caused increase and adrenaline decrease of tonus at all the

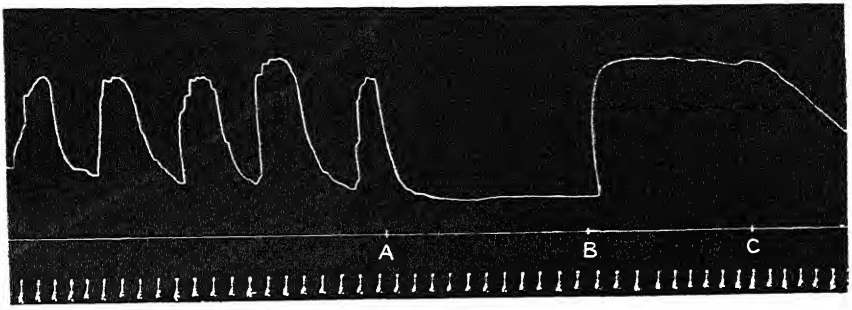

FIG. 4—*Lophius*, intestine, circular muscle. Add drugs to make, at A adrenaline 1/500,000; at B acetylcholine 1/500,000; at C atropine 1/500,000.

dilutions tested (1/1,000,000 to 1/100,000) (seven experiments, figs. 2 and 3).

Isolated circular intestinal muscle of *Uranoscopus* usually showed very marked spontaneous contractions. Addition of acetylcholine caused rise of tonus and disturbed the rhythms of a contracting preparation or initiated rhythms in one which had been at rest. Adrenaline and atropine inhibited all contractions. Pieces of the circular muscle of the intestine of *Lophius* showed slow, rhythmical spontaneous contraction (fig. 4). These rhythms were inhibited by adrenaline or by atropine, and ergotoxine prevented the effects of adrenaline, but did not itself interfere with the movements. Acetylcholine at dilutions of 1/10,000,000 to 1/100,000 caused increase of tonus and the initiation of rhythms, which, however, were less regular than those produced spontaneously.

Three experiments with the rectum of *Lophius* showed that here again acetylcholine caused rise of tonus, adrenaline and atropine a fall, the effect of adrenaline being prevented by ergotoxine (fig. 5).

The gall bladder of both species is large enough to allow of the registration of its movements directly. Slight spontaneous contractions were seen immediately after removal, but these soon died away. The addition of acetylcholine bromide to make 1/100,000 caused immediate rise of tonus and in one case the initiation of rhythmic contractions; adrenaline HCl 1/100,000 then caused marked inhibition (three experiments).

There is therefore great uniformity in the results of these experiments with muscles from different parts of the alimentary tract of *Uranoscopus* and *Lophius*. In all cases acetylcholine causes increase and adrenaline decrease of tonus. As we have seen, the vagus is the motor nerve to the stomach and the splanchnic nerve to the intestine of these fish, so that there is clearly no agreement of the effects of either drug with response to one or the other autonomic nerve.

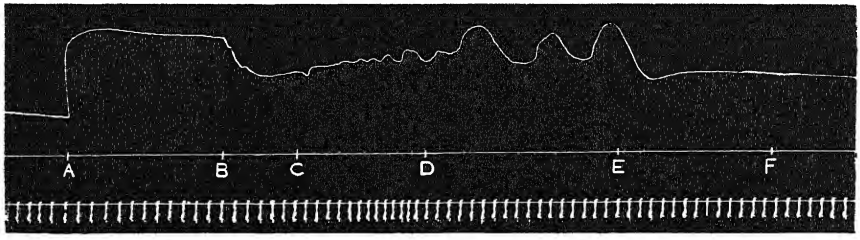

FIG. 5—*Lophius*, rectum, longitudinal muscle. Add drugs to make, at A acetylcholine 1/1,000,000; at B adrenaline 1/1,000,000; at C ergotoxine 1/1,000,000; at D adrenaline 1/400,000; at E atropine 1/400,000; at F acetylcholine 1/200,000.

MOVEMENTS OF THE OVARY AND URINARY BLADDER

The ovaries of the Teleostei differ from those of all other vertebrates in being hollow sacks, directly continuous with the ducts which lead to the exterior. The walls of the ovaries contain circular and longitudinal muscles and receive nerves from the abdominal sympathetic ganglia. Stimulation of the sympathetic chains between the 6th and 10th spinal nerves in *Uranoscopus* or 9th and 11th in *Lophius* was followed by movements of the ovaries, stimulation of one chain causing movements in both ovaries. In five experiments with *Uranoscopus* the ovaries were removed to the bath for registration of their movements. No spontaneous contractions were recorded. After the addition of acetylcholine to make 1/100,000 there was a marked increase of tonus, and in two cases the addition of adrenaline HCl at the same concentration caused distinct inhibition, though this was not seen in the other three cases investigated.

The ovary of *Lophius*, which is very large, gave excellent preparations,

especially from the circular muscles. Slight spontaneous contractions were usually seen when the preparation was first set up, but they rapidly died away. They could be re-started, and maintained for considerable periods, by the addition of acetylcholine bromide at dilutions of 1/4,000,000 to 1/10,000 (fig. 6). Atropine sulphate (1/500,000–1/50,000) sometimes caused a slight fall in tonus, but did not rapidly inhibit the contractions. In only one of the eleven experiments in which adrenaline (1/1,000,000–1/100,000) was tested did it give clear inhibition of the muscle of the ovary, and in three cases its addition was followed by a slight rise of tonus. As will be seen in fig. 6, ergotoxine had no direct effect on the muscle and did not affect the capacity of acetylcholine to produce regular rhythms.

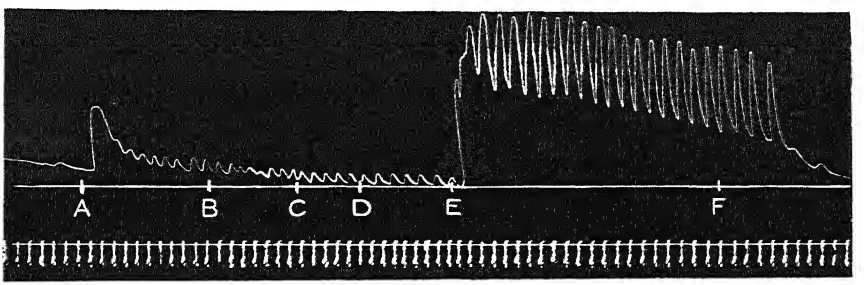

FIG. 6—*Lophius*, ovary, longitudinal muscle. Drugs added to make, at A acetylcholine 1/1,000,000; at B adrenaline 1/1,000,000; at C ergotoxine 1/500,000; at D adrenaline 1/500,000; at E acetylcholine 1/500,000; at F atropine 1/500,000.

In view of the effect of posterior pituitary substances on the uterus of Mammals it might seem likely that they would also cause contraction of the ovary of fish. However, neither Pitocin (2–10 units per 200 ml) nor Pitressin (10–20 units per 200 ml) produced any detectable effects on the preparations (fig. 7).* These observations were made during August and September, when the ovaries are not ripe; it would be interesting to repeat them at other seasons, especially since it is known that the mammalian uterus varies greatly in its susceptibility to Pitocin.

The urinary bladder is large both in *Uranoscopus* and *Lophius*, and receives nerves from the abdominal sympathetic ganglia (fig. 1), faradic stimulation of which causes contraction of the bladder and expulsion of urine (figs. 8 to 12). In *Uranoscopus* the fibres in question run out in the same nerves as the fibres to the ovaries, but in *Lophius* the bladder is innervated by very long nerves of its own, from which excellent nerve-muscle preparations can be obtained. Nerve cells occur scattered along

* The pituitary preparations were kindly supplied by Messrs. Parke Davis, Ltd.

these vesicular nerves and probably also occur in the wall of the bladder itself. So far as could be ascertained by faradic stimulation, the nerve contains only motor fibres to the bladder, but it is possible that inhibitory fibres would be revealed by varying the frequency of stimulation.

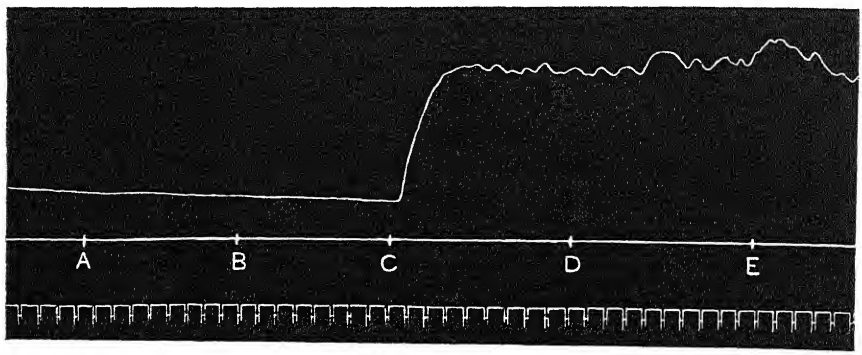

FIG. 7—*Lophius*, ovary, longitudinal muscle. Add drugs to make, at A Pitocin 1 unit per 20 ml; at B Pitressin 1 unit per 20 ml; at C acetylcholine 1/1,000,000; at D adrenaline 1/500,000; at E atropine 1/500,000.

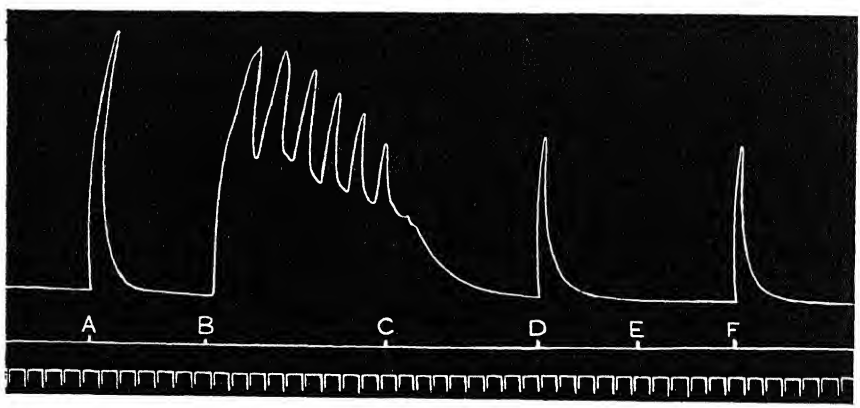

FIG. 8—*Lophius*, urinary bladder. At A, D, and F stimulate vesicular nerve, secondary coil 12 cm. Add drugs to make, at B acetylcholine 1/2,000,000; at C adrenaline 1/500,000, and at E ergotoxine 1/50,000.

The effect of acetylcholine was the same on the bladder as on all the other smooth muscles investigated, namely to cause rise of tonus and the initiation of rhythmic contractions, these effects being obtained in four experiments with *Uranoscopus* and fourteen with *Lophius*, using concentrations between 1/5,000,000 and 1/100,000 (figs. 8, 10, 11, 12). Adrenaline at concentrations between 1/1,000,000 and 1/100,000 usually inhibited

the contractions, but in five out of fourteen cases was without effect on the preparation.

Using the nerve muscle preparation obtainable from the bladder of *Lophius* it was found that ergotoxine 1/200,000–1/50,000 did not inhibit the capacity of the nerve, or of acetylcholine to cause contraction (figs. 11 and 12). Usually the ergotoxine itself was without effect, but in two out of eight experiments it caused contraction (fig. 12).

The influence of atropine (1/500,000–1/50,000) was investigated in thirteen experiments and was found not to abolish the capacity of the nerve to stimulate the bladder muscles. However in some experiments,

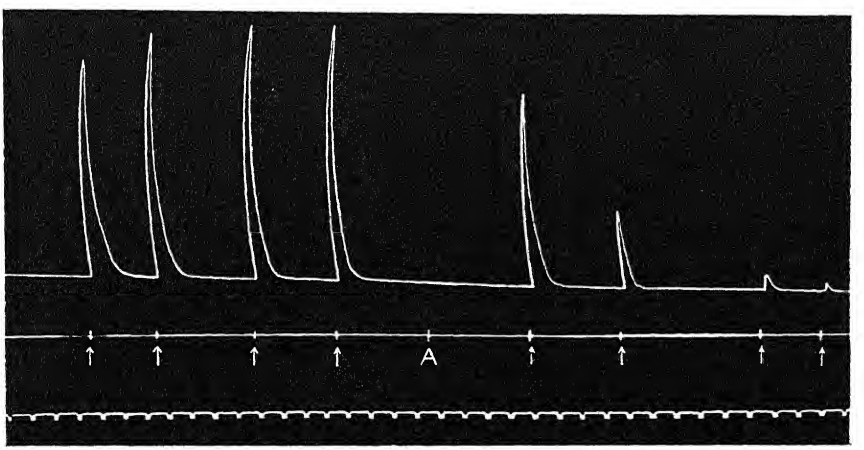

FIG. 9—*Lophius*, urinary bladder. At points marked with arrows faradic stimulation of vesicular nerve, secondary coil at 6 cm. At A add atropine to make 1/50,000.

especially with high concentrations, the threshold of response was slowly raised, so that the response to a given stimulus became smaller (figs. 9, 10, and 12). Eight further experiments showed that atropine does not rapidly inhibit the rhythmical contractions started by acetylcholine (fig. 10). It therefore seems that the action of the acetylcholin on the bladder is " nicotine-like " rather than muscarine-like (Dale, 1914), perhaps indicating that its effect is on the cells in the preparation rather than on the muscles or myoneural junctions.

The bladder and walls of the ovaries, which are embryologically similar, therefore also have similar innervation and reactions to drugs. There is no anatomical or physiological evidence that they have a double motor and inhibitory nerve supply, and none for the existence of a sacral parasympathetic system in these animals.

Discussion

Do these results provide any criteria, either morphological, pharmacological, or physiological by which we can separate the visceral motor nerves of fishes into parasympathetic and sympathetic divisions? The pharmacological reactions are almost uniform for the muscles here studied, acetylcholine causing contraction and adrenaline relaxation in every case,

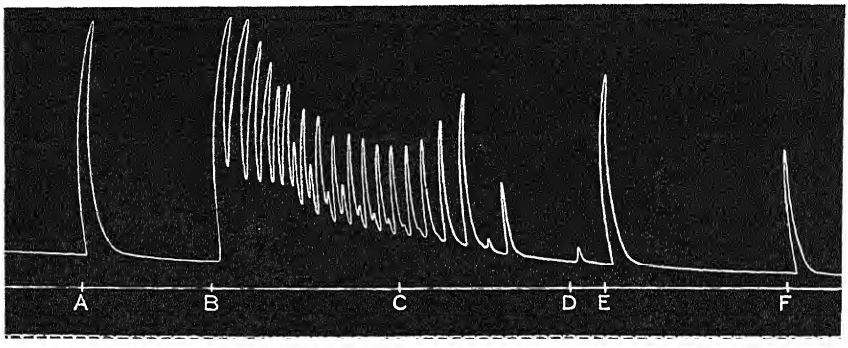

Fig. 10—*Lophius*, urinary bladder. At A faradic stimulation of vesicular nerve, secondary coil 10 cm. At B add acetylcholine to make 1/2,000,000; and at C atropine to make 1/500,000. At D stimulate vesicular nerve with coil at 10 cm, and at E and F with coil at 8 cm.

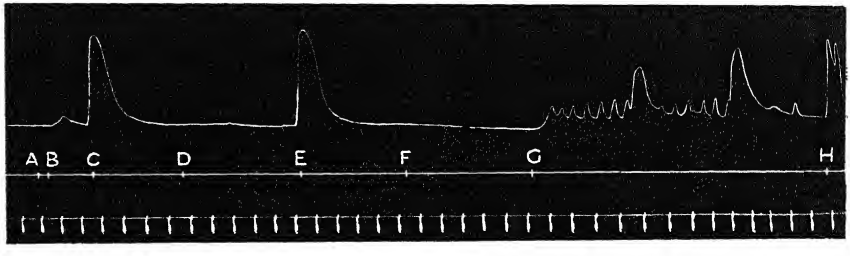

Fig. 11—*Lophius*, urinary bladder. Stimulation of vesicular nerve with secondary coil at 10 cm at A, at 8 cm at B, and 6 cm at C. At D add ergotoxine to make 1/200,000; at E stimulate again, coil 6 cm. At F add adrenaline to make 1/1,000,000, and at C acetylcholine to make 1/1,000,000. At H stimulate, coil 6 cm.

though the effect of adrenaline on the bladder and ovary is not well marked. No separation is therefore possible on pharmacological grounds.

Morphological criteria alone certainly provide no basis for separation of sympathetic and parasympathetic systems (Young, 1933, *b*); and, moreover, muscles with different morphological innervation have been shown above to have similar reactions to drugs. For instance, the muscles

of the stomach, intestine, and bladder are all caused to contract by acetylcholine, although the former receives its motor nerves through the vagus, the two latter through the sympathetic chain.

Further, no evidence is here produced which shows that vagal and sympathetic fibres act antagonistically, though it must be recognized that our knowledge is not yet sufficient to allow us to deny the possibility that such antagonism will be discovered. Until it has been demonstrated, however, in these animals, we certainly cannot use it as a basis for division of their autonomic nervous system.

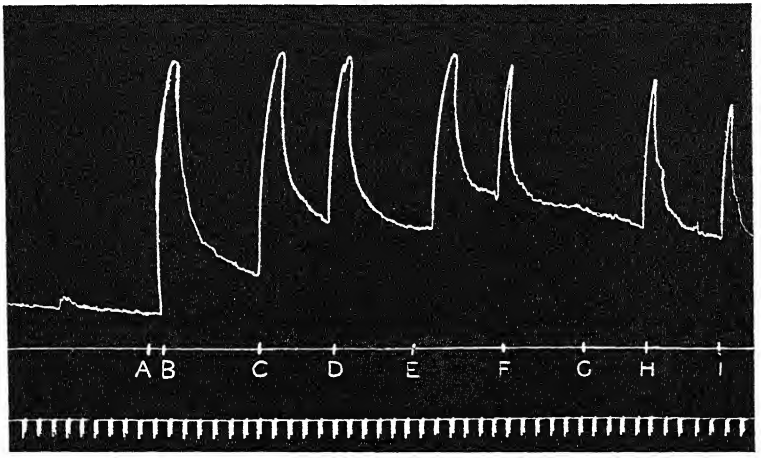

FIG. 12—*Lophius*, urinary bladder. Stimulation of vesicular nerve with secondary coil at 8 cm at A, 6 cm at B. Add drugs to make, at C acetylcholine 1/2,000,000; at D ergotoxine 1/50,000; at E acetylcholine 1/500,000. At F stimulate again, coil 6 cm. At G add atropine to make 1/50,000, and at H and I stimulate with coil 6 cm.

There are therefore, at present, no observations which we can use to give a clear meaning to the concepts of sympathetic and parasympathetic systems in fishes. Until such observations may be made it is advisable to consider the visceral motor fibres of these animals as a single system, within which we can recognize fibres with various functions.

This leads to further enquiry as to the bearing of these experiments on the question of whether autonomic pre- and post-ganglionic fibres exert their effects by means of chemical mediators. It is important to remember that the observation that a chemical substance stimulates or inhibits a preparation *in vitro* does not, by itself, prove that the substance mediates a similar action during life. There are therefore no experiments reported in the present paper which demonstrate the existence of synaptic mediators.

All that has been done is to demonstrate that certain substances which, from experiments on other animals, have been supposed to be such mediators, also produce in Teleosts marked effects at what are, for a cold-blooded animal, low dilutions.

With all these reservations it remains certain that throughout the whole alimentary canal and urinogenital system of Teleosts there is a motor system which can be actuated by acetylcholine and is usually inhibited by adrenaline. This system receives its pre-ganglionic fibres partly through the vagus and partly through the sympathetic chain. The site of action of the acetylcholine in these preparations might be either (*a*) on nerve cells, or (*b*) at nerve endings in the muscles, or (*c*) on the muscle fibres themselves; unfortunately we cannot at present decide between these possibilities. With the bladder the action seems to be " nicotine-like ", and this might be held to favour the first alternative.

Such an interpretation would agree with the results of anatomical investigations (Young, 1931 and 1933, *b*) which have shown that the visceral motor neurons of vertebrates consist fundamentally of a single series. Probably the pre-ganglionic fibres originally ran through the dorsal roots, but have come to pass through the ventral roots in those segments in which the two roots join. The synapse on the path of this single series of visceral motor fibres may possibly have used acetylcholine as its mediator, and this would explain the fact that all the synapses between pre- and post-ganglionic neurons are cholinergic in mammals (Dale, 1933; Feldberg and Gaddum, 1934).

We know still less about the relation of adrenaline to the autonomic nerves in these earlier forms. The facts (1) that adrenaline inhibits the contraction of so many of the teleostean muscles, although they receive motor sympathetic nerves, and (2) that there is no evidence that they receive specifically inhibitory fibres, make one suspect that the adrenaline acts by inhibiting the excitatory mechanism rather than by exciting a specifically inhibitory mechanism. However, it must not be forgotten that the search for inhibitory fibres has hitherto been rather superficial, and that more thorough analysis may reveal their presence in fishes, as it has done in mammals, in places where they are at present unsuspected.

Consideration of the known effects of adrenaline in lower vertebrates does not greatly clarify the question. In Selachians it is known to cause contraction of the stomach (Dreyer, 1928; Lutz, 1931; Nicholls, 1934), but probably inhibits movements of the intestine and rectum. It slows the heart of these animals (Lutz, 1930, Macdonald, 1925, found retardation followed by acceleration), but causes contraction of their arteries and rise of arterial pressure (MacKay, 1931; Wyman and Lutz, 1932).

In frogs Epstein (1932) has shown that it inhibits all parts of the gut, and Adler (1918) that it inhibits the bladder; it is also well known to accelerate the heart and raise the blood pressure of Amphibians.

Taking these observations together with the uniformly inhibitory effects reported in this paper, we see that the effects of adrenaline in lower vertebrates are broadly to raise the blood pressure and inhibit the movements of the viscera. This agrees with the general conclusion of Cannon that in mammals adrenaline serves to prepare the body for defence or attack, and it is to be noted that all of these animals possess chromaffin tissue known to produce adrenaline. A serious difficulty, however, is the slowing of the heart rate of Selachians, which Lutz (1930) has interpreted as an emergency *inhibitory* mechanism, that is to say in the opposite sense to Cannon's interpretation for Mammals. Lutz could only obtain the effect with very large doses of adrenaline, and it is possible that it is not a normal physiological response.

We are still left without much knowledge as to the relation of adrenaline to the autonomic nervous system in these animals, and as to its possible functions in them as a chemical synaptic mediator. We need especially to be certain whether or not there are inhibitory fibres to the viscera, and, if they be present, whether they liberate adrenaline at the periphery. It is to be hoped that with the application to fishes of the more refined methods now used for the study of these subjects in Mammals we shall be able to find the answers to these interesting questions.

Summary

Faradic stimulation of either vagus nerve in *Uranoscopus* or *Lophius* causes contraction of the muscles of the stomach. Stimulation of the splanchnic nerves causes movements in the pyloric caeca and intestine. The splanchnic nerves may also contain motor fibres for the stomach but no effect of the vagus on the intestine could be detected.

No movements of the small intestine or rectum result from stimulation of the hinder sympathetic ganglia, and there is no evidence for the existence of any sympathetic fibres to the alimentary canal except those contained in the one pair of splanchnic nerves.

Acetylcholine causes rise of tonus and the initiation of rhythmic contractions in the muscles of the stomach, intestine, rectum, and gall bladder, whereas adrenaline causes fall of tonus and the inhibition of rhythms in these organs.

The walls of the ovaries and urinary bladder receive motor fibres from

the abdominal sympathetic ganglia, no evidence for the existence of inhibitory fibres to these organs was found.

Acetylcholine causes contraction of the muscles of the bladder and ovaries; adrenaline inhibits the bladder if it affects it at all, it has usually no effect on the ovary.

The motor nerves to the bladder are not rapidly inhibited by ergotoxine or atropine, neither does the latter inhibit the contractions of the bladder which are started by acetylcholine.

These results support the view that in fishes it is not possible by pharmacological, physiological, or morphological criteria to divide the visceral motor nerves into sympathetic and parasympathetic divisions. In particular there is no " sacral parasympathetic " system.

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A Convenient Method for Obtaining Bovine Arterial Blood

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The value, both academic and practical, of accurate knowledge regarding the mechanism of mammary secretion requires no emphasis. Our relative ignorance of what is actually going on in the mammary gland during its activity has been justly lamented from time to time by several writers. Attempts have recently been made to remedy this lack of knowledge, particularly in regard to the nature of the precursors, in the blood as it reaches the gland, of the characteristic milk constituents. It is with a major point in the technique of conducting experiments on bovine blood constituents that this paper is mainly concerned.

An apparently direct method of obtaining information on the question of milk precursors is to make an accurate comparison between the composition of blood entering and that of blood leaving the udder of an actively lactating animal, the samples being taken approximately simultaneously. With due care, it is now possible to obtain useful information by this method. It does not, however, necessarily follow that a change in the amount of any given organic constituent of the blood during its passage through the mammary gland is due to such a substance being withdrawn to form a milk constituent. Part at least may be used up—in fact, some organic material borne by the blood *must* be used up—to provide the energy required for maintenance and secretory activity. The metabolic activity of the gland is evidenced by the marked change in the colour of the blood of the mammary vein as compared with the arterial blood, despite the speed (*see* following paper) with which blood flows through the gland.

In endeavouring to make such comparisons between blood entering and leaving the gland, serious practical difficulties immediately arise. However careful the experimental technique may be, the experimenter is always faced with the fact that even when large quantities of milk are being secreted, the change in composition as between arterial and venous

blood (at least for the great majority of the constituents) is small, and may indeed be very close to the experimental error of the available methods of analysis. We have at present only the vaguest notion of the real speed of blood flow through the gland, but there is no doubt that it is very many times more rapid than the rate of secretion of milk.

Having surmounted experimental and analytical difficulties the conclusion still does not necessarily follow that all of the material taken up from the blood by the gland, even if it is not used for metabolic purposes, actually gets into the milk. Milk and venous blood are not the only exports of the mammary gland. There is also a lymphatic drainage. If our knowledge of the rate of blood flow through the gland is small, that of the rate of lymph flow is infinitesimal. It may well be that the lymph flow is so small in volume that it may be disregarded, but until we have proof of this, the fact that the mammary gland is secreting lymph as well as milk must inevitably render less secure any findings based merely on differences between arterial and venous blood.

Kaufman and Magne (1906) were among the first to make an attempt to relate milk secretion to changes in blood composition. They were able to show that the sugar content of the blood of the mammary vein in the cow was less than that of the blood of the jugular vein. The blood in the jugular vein they considered to be equivalent, as far as the materials involved in milk secretion were concerned, to the blood entering the mammary gland. A similar technique was used by Meigs, Blatherwick, and Cary (1919) who came to the conclusion that the lipin phosphorus of the mammary venous blood was lower, and the inorganic phosphate content higher, than that of jugular blood.

The findings of Kaufman and Magne, and also of Meigs *et al.*, might well have been true, but still might have had no application to mammary secretion, since their assumption that jugular blood is identical with the blood which enters the mammary gland is, as was clearly pointed out by Blackwood and Stirling (1932), untenable. The last workers were the first to use arterial blood for work of this kind, obtained, apparently, without serious damage to or disturbance of the cow, though details of the technique used (which are of first importance in assessing the value of the results) are not given. Their findings differed somewhat from those of earlier workers, in that they came to the very interesting conclusion that the phosphorus of the milk was mainly derived, not as Meigs *et al.* had supposed (using the technique of Kaufman and Magne) from the lipin phosphorus of the blood, but from the acid soluble organic phosphorus, *i.e.*, the phosphoric esters of the arterial blood, and that these compounds might be concerned in the secretion of colloidal calcium

phosphate, thus bringing milk secretion into line with bone formation. However, in a publication which came to hand as this paper was being prepared for the press, Blackwood (1934) has withdrawn this conclusion, and believes that the phosphorus compounds of the milk are probably derived from the inorganic phosphate of the circulating blood.

Our own experiments were planned originally to obtain information as to the precursor in the blood of milk fat. We had first to devise a satisfactory technique for obtaining simultaneous samples of arterial and mammary venous blood.

Blood leaving the udder in the cow can be obtained with relative ease from the abdominal subcutaneous veins which lie superficially on the ventral aspect of the animal and enter the abdominal wall at the "milk wells", and which act as a direct drainage of the mammary gland. These veins are, however, not the only ones which drain the gland, though it is generally believed that the greater portion of the blood leaving the udder traverses them. It is probably legitimate to assume (though at present it is no more than an assumption) that the blood of the abdominal subcutaneous vein represents a mean sample of the blood issuing from the mammary gland at any given moment.

The drawing of arterial blood presents more difficulties. Most of the arteries of the cow are relatively inaccessible, and there is, in veterinary practice, no standard way of obtaining arterial blood samples from this animal. After a number of unsuccessful attempts to obtain blood from the radial artery, we endeavoured to use the caudal artery. Samples of blood were indeed obtained in this way, with somewhat intermittent success, but the effect of this method of bleeding on the animal was so disturbing that it had to be abandoned. We have, however, succeeded in obtaining large quantities of arterial blood with ease and regularity, and without causing any serious disturbance to the cow, from the internal iliac artery by puncture through the rectal wall.

Technique for Arterial Bleeding

A 30-inch length of pressure tubing is tightly fitted with a hypodermic needle of 1 mm bore, the point of which is covered with a small piece of cork to act as a guard against injury to the animal or contamination of the needle with faecal matter whilst introducing it into the rectum. The needle is held in the hand, with the arm already bared and soaped with a fine lather of disinfectant soap to well above the elbow, and carried about 18 inches up the rectum of the cow. The internal iliac artery may be readily palpated at any place from the point where it leaves the dorsal

aorta and leads downwards following the pelvic arch, by gently drawing the fingers backwards and outwards against the rectal wall. After removing the cork guard from the needle with one finger, the rectal and arterial walls are successively punctured and the blood collected in a suitable receptacle from the end of the pressure tube which projects a foot or more clear of the anus. The difficulties are merely those of manipulation. We have found no demonstrable chemical contamination of the blood collected in this way. Bacterial contamination with *B. coli* has been demonstrated to be very small.

The method recommends itself in that the artery which is bled branches to feed the mammary gland, and also that 150 ml of blood or more can be readily obtained in 1 to 2 minutes (or, reckoning from the moment of actual puncture of the artery, in about 20 seconds). In addition, the disturbance to the cow is surprisingly little, possibly even less than that entailed by puncture of the mammary vein, and certainly very much less than that which occurs with any other method known to us for obtaining liberal samples of blood. Out of over 150 arterial punctures made in this way we have only had one serious casualty. In this case the cow had been bled six times within four weeks. The animal died two weeks after the last puncture, a post-mortem examination revealed extensive haemorrhage into the body cavity, complete healing of all the previous punctures except the last, which was badly infected. This was evidently the occasion of the arterial breakdown.

Apart from this there have been no signs of prolonged haemorrhage and no abortions have followed. Several cows have been bled twice in one day and one cow seven times in fourteen days without showing any ill-effects from the procedure. A small haematoma sometimes develops over the point of arterial puncture and may remain for a few days. It has disappeared by the end of 10 days, after which no abnormality can be palpated.

Technique of Obtaining Comparable Samples of Arterial and Venous Blood

Three workers are necessary of whom only one is required to make both the actual venous and arterial punctures. The time is noted when the first person touches the cow, and the whole operation must be complete four minutes later. The first operator grasps the tail of the cow firmly without pulling it, holding it so that it passes in front of the hind leg on the side chosen for mammary venous sampling. This precaution against kicking is not necessary in most cases but adds considerably to the con-

fidence of the second operator who thrusts a 2-mm bore needle into the mammary vein. Care is taken to avoid stasis in making the puncture. The tail of the cow is released and the blood collected without trouble. The first operator may then stand on the other side of the animal holding the vessel for collection of arterial blood in one hand and the other hand placed firmly on the back of the cow. The latter procedure facilitates arterial puncture by preventing the cow from arching her back. Arterial puncture is then made as described above by either the second or third operator depending on which one is collecting the venous sample. Using a 1-mm bore needle for arterial and a 2-mm bore needle for venous punctures the collection of 150 ml of each blood should end practically simultaneously. The individual making the arterial puncture does not handle any of the collecting vessels during the procedure and the first 10 ml of arterial blood are discarded; these two precautions are an additional safeguard against contamination of the sample. We have completed a number of experiments, as outlined above, in under two minutes. The usual time required is, however, about three minutes.

The Proving of the Technique

Before embarking on any lengthy investigation involving its use we made a number of preliminary trials of the technique. Analyses were undertaken of arterial and mammary venous bloods taken, under various circumstances, from a large number of lactating cows. For this purpose about 150 ml of each blood was collected in vessels containing 0·3 gm of potassium oxalate. Iron was determined by a modification of Kennedy's method (1927): haemoglobin by Palmer's (1918) method. Haematocrit values were obtained from heparinized blood. Iron, haemoglobin, and cell volume were determined as an index of possible concentration of blood on passage through the gland. Since milk contains very little iron and no haemoglobin the assumption that both iron and haemoglobin would remain in the blood stream needs no justification. Inorganic phosphorus was determined by Briggs's (1922) method, with suitable modifications for the lipin phosphorus, total phosphorus, and total acid-soluble phosphorus. Total sterol was determined by the method of Bloor and Knudson (1917), and total fatty acid by Bloor's (1928) method. Except for inorganic and total acid-soluble phosphorus analyses, which were done in dulpicate, triplicate analyses were carried out in each case.

The lipoid constituents of the blood and plasma were extracted by Bloor's method. We investigated the criticism of this technique by Mann and Gildea (1933) but we were unable to find any more lipin

phosphorus or total fatty acid after refluxing for one hour than after boiling one minute.

In Tables I–V are shown the results of analysis of some typical arterial and venous blood samples* obtained by the foregoing technique or slight modifications of it:—

(i) Arterial puncture first, venous puncture second. Over five minutes between the two punctures, Table I.
(ii) Venous puncture first, arterial puncture second. Over five minutes between the two punctures, Table II.
(iii) As near as possible simultaneous puncture of artery and vein. Whole experiment completed within three minutes, Table III.
(iv) Venous sample first, followed shortly after by simultaneous arterial and venous samples, Table IV.
(v) Effect of disturbance of the cow on the arterial and venous differences, Table V.

TABLE I—ANALYSIS OF THE CONSTITUENTS OF ARTERIAL AND VENOUS BLOOD WHEN (INTERNAL ILIAC) ARTERY IS BLED ABOUT FIVE MINUTES BEFORE (MAMMARY) VEIN. TYPICAL EXPERIMENT

	Arterial	Venous	Difference A – V
	Mg/100 ml		
Whole blood			
Iron	43·5	43·2	0·3
Inorganic P	4·05	4·36	−0·31
Lipin P	10·8	10·75	0·05
Total fatty acids	274	274	0·0
Plasma			
Inorganic P	4·16	3·96	0·20
Lipin P	8·3	8·25	0·05
Total fatty acids	214	211	3·0

Fig. 1 shows graphically the relationship between the time required to obtain venous blood (*i.e.*, the time between the first operator touching the animal and the first flow of blood from the mammary vein) and the arterial minus venous (" A – V ") difference in level of inorganic phosphate in the blood. All blood samples were taken at approximately

* The plasma figures have a smaller experimental (analytical) error than the corresponding figures for whole blood, and are probably therefore more to be relied upon in drawing conclusions as to changes in composition between arterial and venous blood.

TABLE II—ANALYSIS OF THE CONSTITUENTS OF ARTERIAL AND VENOUS BLOOD WHEN MAMMARY VEIN IS BLED MORE THAN TEN MINUTES BEFORE ARTERY. TYPICAL EXPERIMENT.

	Determinations		Calculations	
	Arterial (A)	Venous (V)	Venous corrected (Vc)*	Difference A − V
	Mg/100 ml			
Whole blood—				
Haematocrit	33·5	34·0		
Haemoglobin	19·8	20·3		
Iron (as Hb)	21·5	22·4		
Cholesterol	183	183	177	6
Total fatty acids	197	194	188	9
Lipin P	9·9	10·0	9·7	0·2
Inorganic P	5·26	5·35	5·17	0·09
Plasma—				
Cholesterol	173	182	176	−3
Total fatty acids	173	153	148	25
Lipin P	6·8	6·8	6·6	0·2
Inorganic P	5·51	6·05	5·85	−0·34

* Vc figures are obtained by determining the relative apparent concentrations of [V/A] from the mean of the haematocrit, haemoglobin, and iron figures, and multiplying the V figure by this factor (in this case 0·967).

TABLE III—ANALYSIS OF THE CONSTITUENTS OF ARTERIAL AND VENOUS BLOOD WHEN MAMMARY VEIN AND INTERNAL ILIAC ARTERY ARE BLED SIMULTANEOUSLY (TOTAL TIME < THREE MINUTES)

	Arterial A	Venous V	Difference A − V
	Mg/100 ml		
Whole blood—			
Haematocrit	39·0	39·0	0
Iron	35·9	35·9	0
Cholesterol	214	213	1
Total fatty acids	276	272	4
Lipin P	10·9	11·1	0·2
Total acid-sol. P	6·79	6·66	0·13
Inorganic P	4·40	4·14	0·26*
Ester P (by difference)	2·39	2·52	−0·13
Plasma—			
Cholesterol	189	189	0
Total fatty acids	267	259	8*
Lipin P	8·1	8·1	0
Total acid-sol. P	4·71	4·40	0·31*
Inorganic P	4·56	4·26	0·30*
Ester P (by difference)	0·15	0·14	0·01

* Figures considered significant.

TABLE IV—ANALYSIS OF THE CONSTITUENTS OF BLOODS FROM THE MAMMARY VEIN AND INTERNAL ILIAC ARTERY WHEN ONE VENOUS SAMPLE (V_1) IS TAKEN PREVIOUS TO THE ARTERIAL SAMPLE (A) AND THE SECOND VENOUS SAMPLE (V_2) IS COLLECTED (FROM THE SAME VENIPUNCTURE) SIMULTANEOUSLY WITH THE ARTERIAL SAMPLE

	Experiment 1—Cow quiet					Experiment 2—Cow somewhat disturbed				
	A	V_1	V_2	$A - V_1$	$A - V_2$	A	V_1	V_2	$A - V_1$	$A - V_2$
	Mg/100 ml					Mg/100 ml				
Whole blood—										
Haemoglobin	25·4	25·7	25·7	−0·3	−0·3	24·6	24·6	24·1	0	0·5
Haematocrit	41	40	41	1·0	0	39·0	40·0	39·0	−1	0
Iron	45·7	45·7	45·7	0	0	43·4	43·8	42·7	−0·4	0·7
Cholesterol	161	159	159	2	2	183	183	184	0	−1
Total fatty acid	216	213	226*	3	−10*	180	174	168	6	12†
Lipin P	8·9	9·0	9·1	−0·1	−0·2	9·6	9·4	9·3	0·2	0·3
Total acid-sol. P ..	6·74	6·54	6·55	0·20	0·19	6·42	6·40	6·30	0·02	0·12
Inorganic P	3·64	3·26	3·35	0·38†	0·29†	3·42	3·28	3·28	0·14	0·14
Ester P	3·10	3·28	3·20	−0·18	−0·10	3·00	3·12	3·02	−0·12	−0·02
Plasma—										
Cholesterol	136	137	137	−1	−1	138	141	137	−3	1
Total fatty acid ..	173	159	169	14†	4†	158	155	158	3	0
Lipin P	5·3	5·4	5·3	−0·1	0	6·3	6·0	5·9	0·3	0·4
Total acid-sol. P ..	4·13	3·50	3·47	0·63	0·66	4·94	4·44	4·52	0·50	0·42
Inorganic P	4·00	3·30	3·40	0·70†	0·60†	4·91	4·30	4·28	0·61†	0·63†
Ester P	0·13	0·20	0·07	−0·07	+0·06	0·03	0·14	0·24	−0·13	−0·21

* Almost certainly experimental error. † Figures considered significant.

TABLE V—THE EFFECT OF DISTURBANCE OF THE COW ON THE DIFFERENCE BETWEEN THE CONCENTRATIONS OF VARIOUS BLOOD CONSTITUENTS IN THE MAMMARY VENOUS (V) AND THE ARTERIAL BLOOD (A). SUMMARY OF SIX EXPERIMENTS (A — V VALUES)

	Cow quiet			Cow disturbed		
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
	Mg/100 ml					
Whole blood—						
Iron	0·10	0·0	0·0	0·0	0·05	−0·03
Cholesterol	0	−2	−3	0	−2	3
Total fatty acids	7	11	5	−2	3	−6
Lipin P	0·15	−0·16	0·00	−0·10	−0·01	−0·08
Total acid-sol. P	0·31	0·33	0·17	−0·01	0·10	0·02
Inorganic P	0·42	0·58	0·28	0·00	0·05	0·22
Ester P	−0·11	−0·25	−0·11	−0·01	0·05	−0·20
Plasma —						
Cholesterol	1	−2	0	0	−1	−2
Total fatty acids	7	5	5	5	0	2
Lipin P	0·0	·0·0	−0·1	0·9	0·04	−0·05
Total acid-sol. P	0·52	0·55	0·48	lost	0·09	0·38
Inorganic P	0·56	0·59	0·38	0·03	0·30	0·00
Ester P	−0·04	−0·04	0·10	—	−0·21	0·38

Experiments 2, 3, and 5 were carried out on the same cow, at the same interval from the last milking, on May 18, 24, 30, respectively.

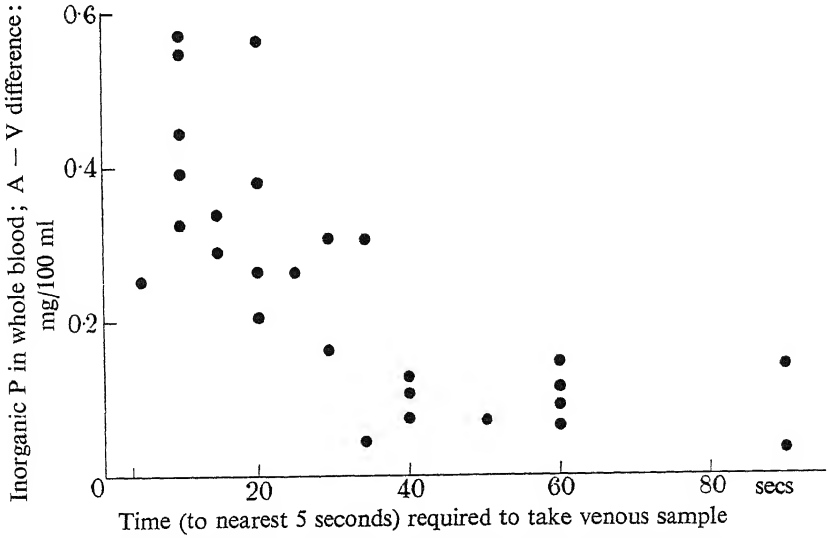

FIG. 1—Relation between the fall in concentration of inorganic P in blood in passing through the mammary gland and the celerity with which the venous sample is drawn. All samples of blood taken at about 10.30 a.m., all cows milked at about 6.30 a.m. All arterial bleedings completed within four minutes.

10.30 a.m., the cows having been milked at about 6.30 a.m. All the experiments were completed within four minutes, this time including the collection of arterial blood following the venous blood sample.

The findings shown in fig. 1, and our experience generally both in the investigation described here and in that to be described in the following paper, lead us to the conclusions that not only is the cow extremely sensitive to disturbance and may react to it by changes in blood composition (compare, for example, the relative lability of the equilibrium in the intact, unanaesthetized cow between blood plasma and tissue fluids as demonstrated by the work of Folley and Peskett (1934)), but also that she is able, by a mechanism not yet understood, but probably effective through vaso-constriction, rapidly to check (at least temporarily) much of the secretory activity of the mammary tissue. The mammary venous blood, apart from its content of sugar and oxygen which have been removed from it for metabolic purposes in its transit through the gland, is then hardly distinguishable in composition from arterial blood. If, therefore, the venous sample is not taken with all speed, there is little doubt that its composition will change during the drawing, giving analytical figures which may result in erroneous conclusions being arrived at regarding the precursors in blood of the milk constituents.

We may summarize as follows the results of the typical experiments tabulated and a large number of others we have made using this technique.

(*a*) Provided the first spurt of venous blood is obtained within thirty seconds of the first operator touching the animal, the whole venous sample within another forty seconds, and the entire period during which both samples are obtained does not exceed four minutes, the results of still quicker technique are inappreciable, *i.e.*, the experiment is probably a good one.

(*b*) No detectable change is apparent in the composition of arterial blood if the above time is not exceeded.

(*c*) If the arterial bleeding is not completed until several minutes have elapsed after the venous bleeding, the venous blood (as judged by iron content, haemoglobin, and concentration of red cells (haematocrit)), frequently appears to be more concentrated than the arterial blood. This is probably due to a progressive dilution of the arterial blood which follows any undue prolonging of the total period of the bleeding.

(*d*) The venous sample should *not* be taken after the arterial sample.

(*e*) Even if the whole procedure is completed in four minutes, serious disturbance of the animal during this time is liable to render the results of much less value. Unless the bleeding technique proceeds with complete smoothness it is advisable (at least as far as drawing any conclusions as

to the precursors in the blood of the milk constituents is concerned) to reject the samples and not waste time on their analysis. In general, disturbance of the cow tends to diminish the small differences in composition between arterial and venous blood, in fact may even render negative A — V values which are positive in the relatively undisturbed animal.

Summary

A technique is described, which it is believed will be of value in the study of the biochemistry of lactation, for obtaining comparable samples of (mammary) venous and arterial blood from the cow. The conditions under which it can be relied upon for experiments on the possible precursors in blood of the milk constituents have been investigated.

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The Precursors in Cows' Blood of Milk Fat and Other Milk Constituents

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There can be no doubt that some constituent or constituents of the circulating blood undergo synthesis or transformation in the mammary gland into milk fat, with its characteristic chemical and physical properties.

Possible origins of the milk fat may be arranged in the following *a priori* order of probability. Milk fat may be formed (1) by transformation of blood fat, *i.e.*, the circulating triglycerides; (2) by resynthesis from blood phospho-lipin (lecithin, kephalin, or sphingomyelin); (3) from blood sugar (providing the glycerol) and cholesterol esters (providing the fatty acid) with the liberation of free cholesterol in the venous blood; (4) from blood sugar (providing the whole of the milk fat by extensive chemical transformations); (5) by some combination of two or more of the foregoing; (6) by some hitherto undescribed transformation.

The well-known suggestion of Meigs, Blatherwick, and Cary (1919) and Meigs (1922) that milk fat is derived from the phospholipin of the blood has held the field for several years. It was supported by Cockefair (1928), who found that the lipin phosphorus in blood taken from the mammary vein of lactating cows at various times after milking increased with the time from the last milking. If the level of phospholipin in the arterial blood remained constant during this period (an unproven assumption) then Cockefair's finding provides indirect support for Meigs, Blatherwick, and Cary's view. Another fact which has been interpreted in favour of this view is that butter fat contains approximately 2 parts of fatty acid of high molecular weight to 1 part of fatty acid of low molecular weight, unlike the fats of blood. It has been suggested that fatty acids of low molecular weight (almost specific to butter fat) are synthesized rapidly in the gland to replace the non-fatty (phosphoric acid–nitrogenous base) substituent on one of the hydroxy groups of the glycerol of the phospholipin. Supporters of the blood triglyceride origin of milk fat have not, however, been wanting. Amongst these may be mentioned Foa (1911), who perfused the surviving mammary gland with a suspension of olive oil in Ringer solution and produced what he believed to be milk

fat, and Petersen, Palmer, and Eckles (1929) who also worked with the perfused, surviving gland. The latter workers found that only small amounts of phospholipin were present in the active mammary gland.

As mentioned in the foregoing paper, Blackwood and Stirling (1932) pointed out a serious source of error in the Kaufman-Magne technique for obtaining blood samples, and that this error would invalidate the interpretation placed on their findings by Meigs *et al.*, who had used this technique before its deficiencies had been realized.

Whilst the work described in the present paper was in progress, Lintzel (1934) published results obtained with goats in which he stated that blood fat, and not blood phosphatide, is the source of the milk fat, and Blackwood (1934) working with cows has also come to the conclusion that blood phosphatide is probably not the source of milk fat.

Using the technique described in the preceding paper for obtaining arterial and mammary venous blood samples practically simultaneously from the undisturbed cow, we believe that we have been able to take the question of the blood precursors not only of milk fat, but also of milk phosphorus compounds and milk sugar a step further towards solution.

Experimental

The work to be described was carried out on cows of the Dairy Shorthorn and Guernsey breeds. All the animals except one belonged to the tuberculin-tested herd of this Institute.

Two groups of experiments were carried out, the first, group Y, during the summer months on four Shorthorn cows giving from 20 to 42 lb of milk per day, and the second, group Z, during the winter months on five Shorthorn and three Guernsey cows giving from 9 to 35 lb of milk per day. The animals were given the usual farm rations during the period of the experiments.

Blood samples were taken at different times after milking from the internal iliac artery and from the abdominal subcutaneous (mammary) vein by the methods and with the precautions already described. In all, over 100 arterial and an equal number of venous bleedings were carried out. With most of the cows several bleedings were made at intervals of several days, each at a different length of time after milking. After each satisfactory bleeding—satisfactory in the sense of para. (*a*), p. 328, of the previous paper—the two samples of blood, venous and arterial, were submitted, side by side, to the same analytical procedure. In the Y experiments the following blood constituents were determined by the methods previously mentioned: iron, cell volume, total fatty acids, total

cholesterol, phosphorus partition; in most samples sugar also was determined by Somogyi's (1926) modification of the Shaffer-Hartman method, and in some chloride by the method of Eisenman (1929).

In the Z experiments, total fatty acid plus cholesterol, inorganic phosphate, and sugar were determined. In order that the utmost care could be given to the awkward fatty acids plus cholesterol analysis, some of the determinations which had been shown by the Y experiments to be of less immediate importance for our purpose were omitted.

In addition, the daily yield and quality (fat and total solids percentage) of the milk were determined throughout.

To avoid lengthy tables of numerical details, and to concentrate attention on the findings which we consider to be of real significance, we have compressed a very large number of experimental results into Tables I and II. (Table I, for example, represents the results of some 460 determinations, most of these being made in triplicate.) The findings are shown as A — V (arterial minus venous) differences, in which a positive sign indicates that the venous blood contains a lower concentration than the arterial blood, *i.e.*, that the constituent in question has been removed to a greater or lesser extent from the blood during its passage through the mammary gland.

It is to be remembered that in all cases except those recorded for the blood sugar, the difference A — V was a small difference between two relatively large experimentally determined figures, each with an experimental error which was a very appreciable fraction of the A — V difference recorded. Fairly wide changes in A — V difference from one experiment to another are therefore to be expected.

Changes in Blood Composition on Passing Through the Mammary Gland

1—*The Y Group of Experiments*—Of the blood constituents examined by us, some show A — V changes well outside experimental error, others show little or no changes within the limits of the analytical technique employed. Those showing definite changes in "satisfactory" experiments are blood and plasma inorganic phosphorus, blood sugar, blood, and plasma fatty acids. Those which do *not* show definite changes in "satisfactory" experiments are blood and plasma total cholesterol, blood and plasma phospholipin, blood and plasma ester phosphorus, blood chloride.

The evidence for the relative stability of the latter constituents will be summarized first, as this finding from the Y experiments was made use

TABLE I—Y GROUP OF EXPERIMENTS

Constituent	No. of experiments	Average arterial concentration	Mean A — V difference	Limits of variation in A — V differences	Standard error of mean of A — V differences	Mean A — V difference expressed as % of the average arterial figure $\frac{(c) \times 100}{(b)}$
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	
			Mg/100 ml			
Blood total cholesterol ..	21	203	−0·22	3·0 to −4·3	0·41	−0·11
Plasma total cholesterol ..	21	196	−0·60	1·0 to −2·8	0·25	−0·31
Blood phospholipin P	21	10·73	−0·10	0·2 to −0·4	0·03	−0·1
Plasma phospholipin P ..	20	7·69	−0·07	0·0 to −0·2	0·005	−0·1
Blood ester P	20	2·88	−0·10	0·0 to −0·4	—	—
Plasma ester P	19	0·29	−0·06	0·2 to −0·4	—	—
Blood chloride	8	284	−0·5	5 to −5	—	−0·18
Blood fatty acids	21	244	4·1	11·8 to −6·5	0·90	1·7
Plasma fatty acids	21	227	4·4	14·0 to −2·3	0·74	1·9
Blood sugar	19	54·7	11·1	6·6 to 17·0	0·66	20·1
Blood inorganic P	21	5·08	0·28	0·10 to 0·50	0·02	5·5
Plasma inorganic P	19	5·22	0·39	0·19 to 0·69	0·04	7·5

of in the Z series of experiments. This evidence may best be presented in tabular form, Table I.

In Table I are shown the results of analyses of blood entering and leaving the mammary gland in a fairly large number of strictly " satisfactory " experiments. In column (*b*) is given the arithmetical mean of the analytical findings for arterial blood. As might be expected there is a fairly large range of variability from one lactating animal to another for all the constituents named. In column (*c*) is shown the arithmetical mean of the A — V differences of each experiment. This mean difference expressed as a percentage of the arterial blood figure gives a fairly sound indication of the tendency to change shown by any given constituent on passing through the mammary gland. If, as is usually accepted, twice the standard error of the mean is taken as the limit of significance, it will be seen from columns (*e*) and (*c*) that the sugar, fatty acids, and inorganic P figures for the A — V difference are all markedly significant.

2—*The Z Group of Experiments*—In these experiments the blood constituents analysed were limited to those which had shown definite changes in the Y experiments, *i.e.*, to total fatty acids plus cholesterol, sugar, and inorganic phosphate, and that in whole blood only. It was evident from the Y experiments that changes in cholesterol and in phospholipin from arterial to venous blood were extremely small* and in fact negligible in comparison with the changes in fatty acids. In the Z experiments, therefore, the total fatty acid plus cholesterol was determined in triplicate on each sample of blood by Bloor's method, and the A — V difference in fatty acid ascertained by subtraction of the venous from the arterial figure on the assumption, which the Y experiments had shown to be legitimate, that the total cholesterol remained unchanged. The results of " satisfactory " experiments are summarized in Table II.

3—*Consideration and Comparison of the Two Groups of Experiments*—Of the various blood constituents, those which show little or no A — V change have probably little or nothing directly to do with the energy requirements of the gland nor with the synthesis of milk constituents, whereas the greatest changes are to be expected in those blood constituents which are either (i) oxidized to provide energy for the functional activities of the gland, or (ii) which are relatively low in concentration in blood but high in concentration in milk, or (iii) which while not themselves appearing in milk, are present in low concentrations in blood and

* Iron and cell-volume changes from arterial to venous blood (not recorded in Table I) were also found to be negligible.

TABLE II—Z GROUP OF EXPERIMENTS

Constituent	No. of experiments	Average arterial concentration	Mean A − V difference	Limits of variation in A − V differences	Standard error of mean of A − V differences	Mean A − V difference expressed as % of average arterial figure $\frac{(c) \times 100}{(b)}$
	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	
		Mg/100 ml				
Blood fatty acids plus cholesterol	19	406	7·2	16 to −3	0·99	3·5*
Blood sugar	23	64·3	10·2	16·9 to 5·0	0·63	16·0
Blood inorganic P	24	5·23	0·28	0·56 to 0·02	0·02	5·4

* (*b*), for the purpose of calculation of this figure of 3·5, was taken as 406 minus 203 (the average arterial cholesterol value as found in the Y experiments), *i.e.*, as 203.

are used in the gland for the synthesis of some major, specific milk constituents.

The percentages shown in the last column of Table II are not dissimilar from those shown in the corresponding column in Table I. Thus two extensive experiments conducted with different groups of cows at different seasons of the year give approximately the same percentage of removal of three of the important blood constituents on passing through the mammary gland. As has been shown, the blood constituents investigated in our experiments can be divided into two categories, (*a*) those that do and (*b*) those that do not alter appreciably in concentration in passing through the udder. This is clear from the results recorded in Tables I and II. It must be pointed out, however, that the A — V figures shown in these tables are averages of individual differences between simultaneous samples of arterial and venous blood taken at various intervals (up to 8 hours) after milking. There is, as will be seen later, a possible relationship between time after milking and the extent of the A — V difference (at least for inorganic P), so that arguments based on averages of this kind must be accepted with caution. Nevertheless, with this proviso, the results may be analysed somewhat further.

The concentration in milk, as in blood, of any given constituent varies considerably from animal to animal, or in the same animal at different times, but we may take as reasonable average figures those given in Table III. Here the concentrations of certain constituents of cows' blood and of milk are shown, together with the summarized findings of the Y and Z experiments.

4—*Rate of Blood-flow through the Active Mammary Gland*—Is it reasonable to conclude from Table III that 850 litres of blood (Y experiments) or 480 litres (Z experiments) are required to provide the fat for 1 litre of normal cow's milk? For a cow yielding 20 litres of milk this would mean for the 1 : 850 ratio a daily circulation of some 17,000 litres of blood through the gland, or nearly 12 litres per minute, an extremely high figure. Although it is true that little is definitely known about circulation rates through the gland, the figure of 12 litres per minute means, with heart rate of 60 per minute, that 200 ml per beat or per second passes through the udder. Even with the lower figure of 1 : 480 the circulation rate through the gland would be some 9000 litres per day, or 6 to 7 litres per minute, or of the order of 100 ml per heart beat. Data with regard to the total output of the left ventricle per beat of the heart of the lactating cow do not seem to be on record. Taking the adult human output per beat of some 60 ml in its relationship to the total

blood volume of some 5 litres as a guide, and using Turner and Herman's (1931) figures for the blood volume of the dairy cow, we may make a guided guess that the output per beat of the heart of the lactating cow is of the order of 400 ml. It is clear that there must be a large circulation through the mammary gland, but whether as much as one-quarter of the heart's output passes through it as would apparently be demanded, by the 1 : 480 ratio, for a fairly heavily milking cow must remain for the time being possible but unproved.

TABLE III

Constituent	Concentration in whole blood (*a*)	Average A – V change in mammary gland (*b*)	Concentration in milk (*c*)	Ratio *a* : *c* (*d*)	Ratio *b* : *c* (*e*)
		Mg/100 ml			
Cholesterol	203	−0·2	14*	1 : 0·07	—
Phospholipin P	10·7	−0·1	6·5	1 : 0·6	—
		Y experiments			
Sugar	55	11·1	4800	1 : 85‡	1 : 430
Total fatty acids ..	244	4·1	3500	1 : 14	1 : 850
Inorganic P	5·1	0·28	68†	1 : 13	1 : 240
Total P.....	—	(0·28)	104†	—	1 : 372
		Z experiments			
Sugar	64	10·2	4800	1 : 75‡	1 : 470
Total fatty acids ..	203	7·2	3500	1 : 17	1 : 480
Inorganic P	5·2	0·28	68†	1 : 13	1 : 240
Total P.....	—	(0·28)	104†	—	1 : 372

* Denis and Minot (1918).

† Graham and Kay (1933).

‡ Assuming that 1 gm glucose = 1·0 gm lactose (actually 1 gm glucose = approximately 0·95 gm lactose).

The work of Jung (1933) on two lactating goats, carried out by direct ("stromuhr") measurements of blood flow in the mammary artery, points to a smaller rate of circulation relative to the milk secreted, namely, 70 volumes of blood to 1 of milk in the first animal (173 litres of blood per day), and 150 to 1 in the second (180 litres of blood per day). These ratios must both be regarded as erring well on the small side of the true physiological ratio, since the experiments, of a somewhat drastic kind, were done without anaesthetic, and there is little doubt that painful

stimuli result in a vaso-constriction in the gland. More weight may probably be placed on the much higher rate for blood flow in the goat (similar to that found by us for the cow) indicated recently in several experiments, by Lintzel (1934, *a*) who finds that for 1 litre of milk 540 litres of blood must pass through the gland to provide the milk fat, and 450 litres to provide the milk protein. He calculates that 1200 litres of blood per day must pass through the udder of a high yielding goat, *i.e.*, some six or seven times the circulation rate suggested by Jung's findings.

In examining this apparently high rate of blood flow disclosed by our own experiments, there are three considerations to be borne in mind. In the first place it is possible, in fact almost probable, that the blood fatty acids are not the only source of the milk fatty acids. The relatively high proportion of short-chain fatty acids in milk fat [Dean and Hilditch (1933) find some 20% (molar percentage) of acids up to C_{12}] may, it is true, be derived by selection from the blood fatty acids, but it seems at least equally likely that they are synthesized from some non-fatty constituent of blood by the mammary gland. In the second place we have averaged, for purposes of our calculations, all our experiments, from good, average, and indifferent yielders, a procedure which is not entirely satisfactory. In the third place it is not unlikely that our mean A — V figure for blood fatty acid is too low. Even a small error in experimental technique in obtaining the blood samples leads, in our experience, to a *diminution* in the A — V difference in the key blood constituents (fatty acids, inorganic P, and to a lesser extent, sugar), and it is to be noted that, with probably improved technique in the Z experiments, the average A — V difference for fatty acids is increased.

On the other hand, with a reasonable number of figures at our disposal we find no correlation between the total milk-fat yield and the A — V differences for blood fatty acids. It is, of course, not necessary that there should be any such correlation; a higher yield of milk and milk fat may simply be associated with a higher rate of blood flow through the gland without an increase in the percentage removal of any of the blood constituents. Nevertheless, results (insufficient in number to be entirely convincing) are available which indicate the likelihood of a correlation between A — V differences for blood sugar and milk yield (*i.e.*, yield of lactose, since normal milk has a fairly constant lactose percentage), fig. 3.

It is somewhat encouraging that, despite the accumulation of assumptions and errors (at present almost inevitable) which must interfere with the drawing of any really clear-cut conclusion from Table III, the ratios for sugar, total fatty acids, and total phosphorus shown in the last column

are of the same order. There can, we think, be little doubt that the circulation of blood through the cow's mammary gland must be very rapid, possibly of the order of 300–400 litres for every litre of milk secreted. At present, direct information on this important point, though urgently needed, appears to be entirely lacking.

It is to be expected that the very large volume of blood continually passing through a heavily milking gland would require a larger cross-section in the veins necessary for drainage than the cross-section required in a gland of lesser output. This is, in fact, to be observed; Aldrich and Dana (1917) showed that there was a high degree of correlation between the diameter of the mammary vein and milk production, while Garner (1932) has recently confirmed this correlation between the size of "milk well" (the point of entry into the body wall of the mammary vein, along which most of the venous drainage of the gland is believed to be effected) and the milk yield.

5—*Possible Loss of Water from the Circulating Blood*—If the loss of water from the blood on passing through the gland is of the order of one part in 400 only, it is unlikely that it will be detectable by the ordinary methods of blood analysis, few of which are accurate to the required degree. It is, however, interesting to notice in Table I that the cholesterol and phospholipin figures indicate for column (*d*) (in which the averages for a large number of A — V figures are shown) that these constituents apparently increased very slightly on passing through the gland. This may possibly be an index of slight concentration of the blood owing to abstraction of water from it on passing through the udder, associated with the fact that cholesterol and phospholipin are less in concentration in milk than in blood.

No significant difference between either the chloride or the iron content of arterial and venous blood was shown in a small series of experiments carried out on the same samples of blood (chloride—Eisenman's (1929) method; iron—Kennedy's (1927) method).

Changes in Specific Precursors

1—*Inorganic P Changes*—The relationship between the A — V differences and the time elapsing between the last milking and the taking of the blood samples is shown for eight different individual cows in fig. 1. It will be noticed that there is a tendency for this difference to increase in the first hours after milking to reach a peak after four hours, and then to diminish to a considerably lower figure after the gland has filled. Until

we can measure the rate of mammary blood flow at various intervals after milking, it is not possible to say whether the apparent peak of inorganic P loss from the blood is indicative of highest secretory activity at this time, or merely of slowest blood flow.

It may be concluded from the results given in Table I and in fig. 1 and from similar findings not detailed here that in all " satisfactory " experiments (*a*) there is a clear-cut fall in the inorganic phosphate of blood in traversing the mammary gland (confirming Lintzel (1934, *b*) and Blackwood (1934)); (*b*) the A — V difference appears to increase up to about the fourth hour after milking, after which it diminishes; (*c*) cows giving

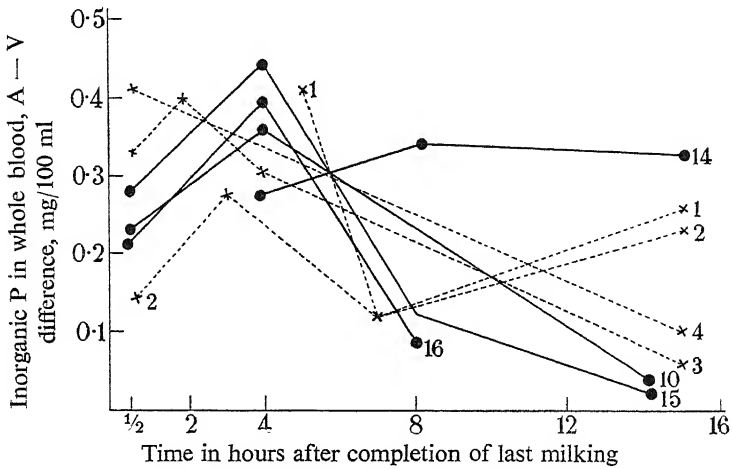

FIG. 1—Relation between the length of time since the last milking and the fall in concentration of inorganic P in blood in passing through the mammary gland.

large quantities of milk show, with increasing time after milking, *i.e.*, as the udder becomes engorged with milk, a more rapid falling off in their A — V difference than less productive cows.

Since the other " phosphorus fractions " in the blood are found to vary relatively little as between arterial and venous blood (in fact there seems to be a tendency for the " phospholipin " and " ester "* phosphorus to increase very slightly on passing through the active gland (*see* Table I)) it may be concluded that the phosphorus compounds of milk must come, in the main, from the *inorganic phosphate* of the blood reaching the mammary gland and not to any appreciable extent from the other phosphorus containing compounds in this blood.

* The accumulated errors on the plasma ester P determination are so relatively enormous (the figure being a difference between two approximately equal quantities, each with an appreciable experimental error) that it cannot be taken too seriously.

2—*Sugar Changes*—In all the "satisfactory" experiments, without exception, there was a fall, usually a marked fall, in blood sugar in passing through the gland. It may be assumed safely that all, or most of this fall, was due to glucose being taken out of the blood for one or both of two purposes, (*a*) to satisfy the energy requirement of the gland; or (*b*) to enable lactose to be secreted in the milk. That the energy requirements of the actively secreting udder are not small is clear from a simple inspection of the blood—unmistakably venous in colour—leaving the mammary gland. There is little doubt that some carbon containing substance is being oxidized in substantial quantities during secretion. Until the rate of blood flow through the gland during the normal process of activity can be determined, the quantity of such carbon-containing substance or substances used for metabolism cannot be even approximately decided. It is likely, however, to be fairly large.

Normal milk from the animals of our herd contains about 4·8% of milk sugar. If lactose is derived from the glucose of the blood which disappears during the passage of blood through the udder, then some 400–500 litres of blood must pass for every litre of milk produced. If, however, some of the sugar which disappears from the arterial blood is used for gland metabolism, the quantity of blood required to supply *both* the metabolic glucose and the glucose required for lactose synthesis would be considerably increased. If this is true, we are again faced with a very high figure for the circulation rate through the gland. It seemed to us possible that the blood sugar level might well be the limiting factor either in quantity or quality of milk secretion. On examining our data from this point of view two correlations were observed. In the first place there was, in a number of the animals, a reasonably close correlation between the arterial level of blood sugar and the quantity of sugar removed on passing through the gland (A — V difference). Thus with two normal Shorthorns, examined at intervals over some months the curve shown in fig. 2 was obtained, giving a correlation coefficient of 0·935, the corresponding value of "t" (Fisher, 1934) being 7·15 and "P" being much less than 0·01. With four Guernseys examined similarly the correlation coefficient was 0·747, with "t" of 2·74 and "P" of between 0·05 and 0·02. This points to a close degree of correlation between the arterial blood sugar and the A — V difference, and establishes that the probability that this degree of correlation should appear fortuitously is very small. It may be taken that in the majority of apparently normal cows, there is a definite relationship between the concentration of sugar in the arterial blood reaching the gland and the amount of sugar in milligrams per unit volume of blood that the gland takes out of the blood.

The other relationship which seemed likely from our results, but not yet proved, was that between the milk yield (average weight per day) and the average amount of sugar taken out of the arterial blood by the gland. In fig. 3 the ordinates show the mean figure of all the differences

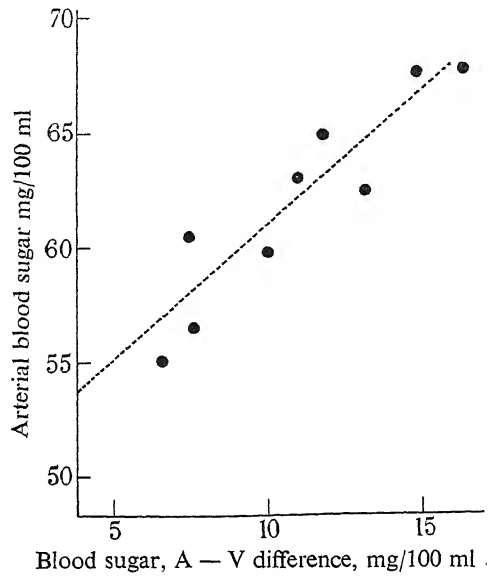

FIG. 2—Relation between concentration of sugar in arterial blood and the fall in concentration of sugar in blood in passing through the mammary gland.

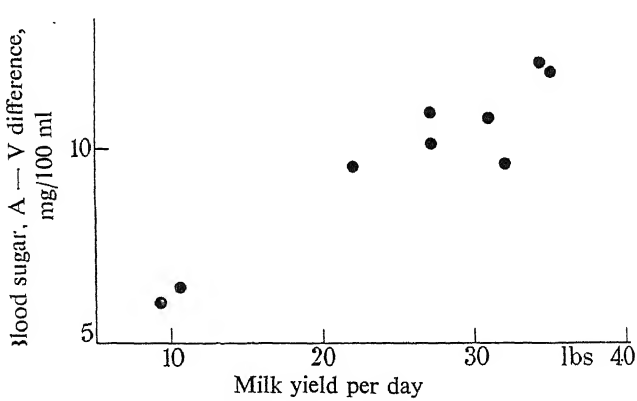

FIG. 3—Relation between milk yield and the fall in concentration of sugar in blood in passing through the mammary gland. (Shorthorns only.)

between the arterial and venous samples taken from each individual cow during the (relatively short) period for which the average daily milk yield is given in the abscissae. Before this apparent correlation can be taken as completely established, more data of a similar kind are required.

It has recently been found by Graham (1934, *a*, *b*) that either thyroid gland feeding, or thyroxine administration, leads to a rise in the percentage and in the total amount of milk fat secreted in a given time by the cow's mammary gland. From further experiments of Jones and Graham (1935) and of Folley and White (*see* following paper) it is now known that thyroxine administration leads to (i) an increase in the blood sugar; (ii) an increase in the milk volume; (iii) an increase in the total and percentage of fat secreted in the milk; (iv) an increase in the non-fatty solids of the milk; and (v) an increase in the milk sugar. These changes indicate that the effect of the thyroid hormone is to increase both the quantity and the quality of the milk secreted. Is it possible that this effect is due essentially to the increase in blood sugar which is produced by the hormone? If some method could be devised for maintaining the blood sugar at a high level for some days without seriously disturbing the cow, it might be possible to answer this question.

We have carried out a few experiments in which the blood sugar of lactating cows has been artificially raised for several hours by direct administration, either subcutaneously or continuously intravenously of glucose-saline solutions. So far, no increase either in milk volume or milk quality has been produced in this way, possibly owing to the experimental difficulties which are by no means trivial. Whitnah, Riddell, and Hodgson (1933) by pumping glucose solutions into the stomachs of lactating cows have brought about, by perhaps less unphysiological means, large though temporary increases in the blood sugar of these animals, changes which they find to be associated with temporary rises in the lactose content of the milk. The work of Gowen and Tobey (1931) on the effect of inanition, of insulin and of phloridzin administration on the lactating cow has shown that both the total quantity of milk and the quantity and percentage of lactose secreted in a given time are quickly diminished by a slight lowering of the blood sugar level, however this lowering is brought about.

3—*Fatty Acid Changes*—The analytical methods for lipin constituents of blood, particularly the micro-methods applicable to relatively small quantities of blood, are not yet entirely satisfactory. Since the lipin changes between arterial and venous blood may be expected to be small, it is probable that the experimental error on any methods used is an appreciable fraction of the possible A — V difference.

After experiments with other methods, which were set aside owing to either relatively large variations between duplicates, or the inordinate time or excessive manipulation required to produce even a doubtful

result, we decided to use Bloor's method (1928) of extraction and, after saponification of the extract with sodium ethylate, to carry out his dichromate oxidation of the fatty acids + cholesterol. In the Y experiments, cholesterol was determined directly on an aliquot of the extract (Bloor, Pelkan, and Allen, 1922), and this figure was subtracted from the dichromate oxidation figure to give " total lipin fatty acids ". (In the Z experiments, cholesterol was not directly determined.)

There is thus some accumulated error in the " total fatty acid ". Nevertheless, in " satisfactory " experiments, the same order of A — V difference is observed, and the two separate groups of experiments, Y and Z, tell the same story. The A — V differences in fatty acids are somewhat irregular. We have plotted these differences against time after milking, and no clear relationship is to be observed.

Strictly, all that it is permissible to deduce from our experiments on fatty acid changes is (*a*) that the total fatty acid of the blood diminishes during its passage through the mammary gland, and (*b*) that the phospholipin of the blood suffers little change in its passage through the gland. If we connect the fall in " total fatty acid " with the simultaneous secretion of fat into the milk, we must do so with caution until we know what material is actually being oxidized for energy purposes in the gland.

Cholesterol esters were not determined separately and we cannot therefore state definitely whether the loss in total fatty acids on passing through the gland is to be ascribed wholly, in part, or not at all to changes in the quantity of fatty acid combined with cholesterol. According to Lintzel (1934, *b*) the cholesterol esters do not provide the fatty acids for secretion of milk fat in the goat.

Summary

With the provisos mentioned in the text, certain conclusions may be stated.

(i) The fat of cows' milk is derived in the main from the non-phospholipin fatty acids of blood, *i.e.*, probably from those of neutral fat. Its derivation from the fatty acids of cholesterol esters is not, however, entirely excluded. Phospholipin is not the blood precursor of milk fat.

(ii) The phosphorus compounds of milk are derived in the main from the inorganic phosphate of the blood plasma. The phosphoric esters of blood are almost certainly not the immediate precursors of the milk phosphorus compounds. Nor is the phospholipin of blood the source of any appreciable quantity of the phosphorus of milk. The rate of uptake of inorganic phosphate from the circulating blood by the mammary

gland is not constant during secretion but depends on the time that has elapsed since the previous milking. Cows giving larger quantities of milk show, as the gland fills with milk, more definite falling off in the amount of inorganic phosphate taken out of unit quantity of blood than do cows producing smaller quantities of milk.

(iii) Considerable quantities of sugar disappear from blood during its passage through the active udder. The amount of sugar taken out of unit volume of blood during its passage through the gland is related to the level of sugar in the arterial blood, and probably also to the volume of milk secreted.

(iv) The number of volumes of blood required to produce one volume of milk is of the same order whether calculated from fatty acid changes, inorganic P changes, or sugar changes between arterial and venous blood. (Implicit in this conclusion is the assumption that mammary venous blood is characteristic of the entire venous drainage of the gland and that the lymphatic drainage can for the present purpose be regarded as negligible.) Our results point to a very rapid circulation through the active mammary gland, possibly of the order of $1\frac{1}{4}$ gallons of blood *per minute* for a cow yielding 4 gallons of milk per day.

Where we have covered the same ground, our findings agree, in the main, with those obtained by Lintzel for goats and support Blackwood's (1934) view regarding the part played by phospholipin in fat secretion in the cow.

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The Effect of Thyroxine on Milk Secretion and on the Phosphatase of the Blood and Milk of the Lactating Cow

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Introduction

The concentration of phosphatase* in the blood serum of normal men differs very little from one adult individual to another. The same is true of a number of other species though in certain animals, such as the dog, the normal variation between individuals is somewhat wider than in man (*see* King and Armstrong (1934)). On the other hand, in apparently normal cows of the herd of the National Institute for Research in Dairying, it has been found that while the serum phosphatase of any one individual remains within comparatively narrow limits over a period of months, the values for different cows vary widely. Indeed, the level of phosphatase in the blood serum of some cows has been found to differ from that of others by factors as great as 12.

In seeking to account for this curious phenomenon, we were led to consider hyperthyroidism in man, which was found by Kay (1930) to

* The word "phosphatase" is used throughout this paper to denote the phosphomonoesterase with a p_H-activity optimum in the neighbourhood of p_H 9 (*see* Folley and Kay (1936, *a*)).

be accompanied by increased serum phosphatase. It occurred to us that a possible explanation of the wide variations in the serum phosphatase levels of different cows might be found in individual differences in thyroid activity, particularly since Scoz and Marangoni (1934) had shown that single injections of thyroxine into dogs lead to considerable transient increases in serum phosphatase. Accordingly we decided to study the changes in serum phosphatase brought about by injecting thyroxine into lactating cows characterized by widely different initial values of serum phosphatase. We considered that if our conjecture was correct, not only should the level of serum phosphatase rise as a result of the injections, but the response should be greatest in those animals whose initial levels were lowest. In view of the well-known relationship between serum phosphatase and the metabolism of calcium and phosphorus, it seemed desirable to study serum calcium and inorganic phosphate concurrently.

A phosphatase with properties identical with those of the blood serum enzyme is known to occur in milk (*see* Graham and Kay, 1933–4). It is conceivable that the enzyme in milk is derived directly from that in blood, though since the phosphatase activity of normal milk is usually considerably higher than that of normal blood plasma, some concentration of phosphatase would have to be effected by the mammary gland. If this is so, and further, if the mechanism whereby passage of the enzyme from blood to milk takes place is relatively simple, we should expect that variations in the concentration of phosphatase in the blood would be reflected, at least qualitatively, in the milk. If, on the other hand, the milk phosphatase is derived from phosphatase stored in, or even synthesized by, the cells of the mammary glands (and Folley and Kay (1935) have shown that mammary tissue is strikingly rich in this enzyme), no such relation is necessarily to be expected. We therefore thought it of interest to make collateral measurements of the concentration of phosphatase in the milk of the cows used in this experiment, since if the absence of a positive correlation can be established, the hypothesis of the origin of milk phosphatase from the blood serum becomes untenable.

The use of lactating animals as experimental material in the projected investigation just outlined, afforded an excellent opportunity of combining with it a study of certain aspects of the relationship between the thyroid gland and milk secretion, a hitherto comparatively neglected field of research in which interesting preliminary results had been obtained by other workers. Graham (1934, *a*) found that feeding desiccated thyroid gland to cows, both normal and thyroidectomized, in declining lactation, brought about an increase in both milk and milk-fat secretion. If thyroid was withdrawn from the diet of thyroidectomized animals, an

immediate regression of milk and milk-fat secretion resulted. Thyroid feeding was found to be effective only in declining lactation, it having little effect on milk secretion during the four to six weeks following parturition when milk secretion gradually rises to a peak. Graham (1934, *b*) later was able to show that similar results could be obtained by parenteral administration of thyroxine to lactating cows.*

Jack and Bechdel (1935) found that injections of thyroxine sufficient, according to their calculations, to increase the basal metabolic rate of their cows by 10% were without appreciable effect on milk composition, though with injections estimated to cause a 30% increase in the basal metabolic rate, an increase in milk yield could be detected in declining lactation.

Graham's results led him to the view that the thyroid hormone neither primarily controls lactation nor forms one of the chain of controlling hormones. Indeed, he suggests that the thyroid affects merely the plane of milk secretion and does so through its action on the metabolic rate. Jack and Bechdel take quite another view. Setting aside the evidence, admittedly inconclusive, which Graham adduces in support of his theory, they suggest that thyroxine influences milk secretion indirectly by stimulating the anterior lobe of the pituitary to increased production of the lactation hormone (*see* Folley (1936)).

It may be noted that in the experiments considered above, each type of treatment was applied only to single animals at one time, and that no control animals were used. In view, therefore, of the interest of these preliminary findings and their ultimate importance for our knowledge of the intimate mechanism of lactation if confirmed and extended, we considered it desirable to enlarge our projected experiment to include a study of the secretion of milk, milk fat, and milk non-fatty solids by the cows participating, the experiment being so designed that close comparison could be made between groups of experimental animals and controls. We also considered it desirable to estimate the precision of the results by statistical methods.

Experimental

Experimental Animals—Two groups of Dairy Shorthorn cows of the Institute herd (comprising registered and "grading-up" cattle) were used. Table I gives the age and weight of each animal together with the number

* Graham and Jones (quoted by Jones, 1935), in hitherto unpublished experiments, observed an increase in the non-fatty solids content of the milk of cows receiving thyroxine injections, which Jones (1935) has ascribed, at least in part, to an increase in lactose content.

of weeks that had elapsed between parturition and the beginning of the experiment. This latter quantity denotes the stage in the lactation cycle when the experiment began. The groups were made up so as to be as nearly comparable as possible with regard to the ages, stages of lactation, and weights of the cows comprising them.

TABLE I

		Age years	No. of calf	Weeks in milk	Weight kilos
Thyroxine injected	Lily 3	8½	6	13	564
	Red Rose	6½	4	20½	600
	Dora 4	5¾	4	19	562
	Wistaria 4.....	6½	4	24½	562
Control	Peach 3	8¾	6	10	551
	Winsome 2 ..	11½	9	23½	610
	Flora 16	5¾	3	27½	635
	Portia 3.....	4¾	2	17½	629

All the experimental animals were kept under the same regime, being turned out to pasture except when brought into the cowshed for milking. They were milked twice daily at about 7 a.m. and 4 p.m., the total yield from each cow being determined to 0·25 lb on a spring balance.

Thyroxine Injections—Each cow of the experimental group was given a daily subcutaneous injection of 10 mg thyroxine in the region of the shoulder for fifteen consecutive days, the injections being given immediately after the afternoon milking. The injection period was preceded and followed by control periods of eight days. There was evidence that the after-effects of the injections had not completely disappeared by the eighth day after the last injection but unfortunately, since the animals were required for another experiment, the analyses could not be extended. Measurements of morning and evening milk yields were, however, available for a further ten days, during the last nine of which no after-effects were detectable.

Thyroxine-sodium B.D.H. was used, the rather insoluble material being brought into solution with the minimum amount of caustic soda. This solution was neutralized with HCl to the point of re-precipitation and brought to volume with distilled water so that 1 ml contained 1 mg thyroxine.

Blood Analyses—Twice weekly throughout the experiment, 125 ml blood samples were taken from the abdominal subcutaneous veins of all

cows. Blood samples were always taken at approximately 9.30 a.m. The blood was allowed to stand some hours at 37° and then at room temperature. Twenty-four hours after the samples were taken, the sera were removed from the clots, centrifuged, and the analytical work upon them started.

Total calcium was determined by the Clark and Collip (1925) modification of the Kramer and Tisdall (1921) method, using N/200 permanganate, and with the modification that oxalate precipitation was allowed to proceed for 24 hours, inorganic phosphate by the Briggs (1922) method, and phosphatase by the procedure of King and Armstrong (1934), the incubation time being 30 minutes at 37° C.

Milk Analyses—The milk yield from each cow at both milkings was determined daily and at the same time samples were taken for the determination of the fat content by the Gerber method, and of the total dry matter by evaporation at 105° C. The percentage by weight of non-fatty solids in the fat-free milk serum (*see* Bartlett, 1933–4) could be calculated from these data.

Determinations of phosphatase in morning and afternoon milk of all cows were made twice weekly by the method of Kay and Graham (1933–4), the samples for these estimations being taken on the day following those on which blood samples were taken.

Clinical Observations—No facilities were available for the measurement of the metabolic rate of cows. For information regarding the effect of our administration of thyroxine on the metabolic rates of the experimental animals, we were therefore forced to rely on measurements of body temperature, pulse and respiration rates. These were made twice weekly throughout the experiment immediately after the afternoon milking.

Results

Pulse Rate, Respiration Rate, Body Temperature—The differences between the mean pulse rates for injected and control groups are plotted in fig. 1. The dispersion of the individual results is indicated by the vertical lines, the whole length of each of which is twice the probable error of the corresponding point.* From the figure it is evident that the reaction to thyroxine first becomes evident two days after the start of the injections. By the sixth day the mean pulse rate of the injected group

* Throughout this work, in calculating standard deviations, probable errors, and standard errors of means, we have always used Jeffreys's (1932) correction which is important where the number of observations is small.

exceeds that of the non-injected group by 19 beats per minute (probable error equals 2). The mean increase in pulse rate due to thyroxine is 22 beats per minute (probable error approximately 1·3). After cessation of injections no diminution in response is evident for three days, but all difference in mean pulse rate between the two groups has disappeared by the seventh clear day.

No such clear result emerged from the observations on respiration rate and temperature, since here the standard errors were rather large. There were, however, indications that thyroxine administration had led to some degree of enhanced respiration and hyperthermia.

Blood Serum Phosphatase—The problem of analysing the serum phosphatase results is complicated by the wide difference in initial serum phosphatase values between different animals. Thus the highest and lowest initial levels of phosphatase in the sera of the cows used in this experiment were in the ratio of 12 : 1. On this account, the group arithmetic mean is not a good index of the behaviour of the serum phosphatase values of a number of cows, since the behaviour of arithmetic means of widely divergent numbers is largely a reflexion of the behaviour of the larger of the quantities from which they are calculated, while that of the smaller ones, though equally relevant to the problem, is to some extent masked.

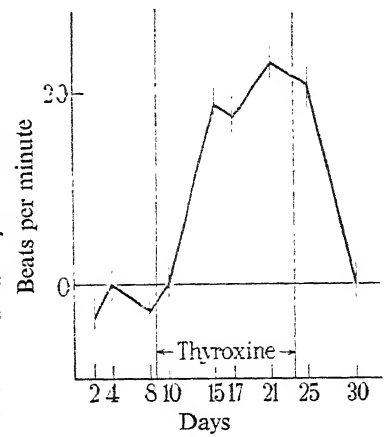

FIG. 1—Difference between mean pulse rates of thryoxine injected and control groups of cows. The whole length of each vertical line represents twice the probable error of the determination plotted at its mid-point.

We have accordingly taken group averages not of the absolute serum phosphatase values, but of the relative values which for any given cow are expressed as percentages of the mean phosphatase activity of its serum over the initial control period. The same method of expression is used also in dealing with the phosphatase present in the milk, and these values are described as "relative" blood serum and milk phosphatase activities respectively.

The difference between the mean relative blood serum phosphatase activities for the two groups of cows is plotted in fig. 2. The effect of thyroxine is to raise the level of the serum phosphatase by 13% with a standard error of 4·5 units of percentage. After the injections had

ceased, the mean relative phosphatase activity of the injected group sank below the level of that for the control group, the difference being −28% by the seventh day after the end of the injection period, and there was no sign of return to parity with the values for the control group at the end of the experiment.

No correlation could be found between the initial level of serum phosphatase and the magnitude of response in serum phosphatase to thyroxine nor any other individual characteristic of the cows investigated.

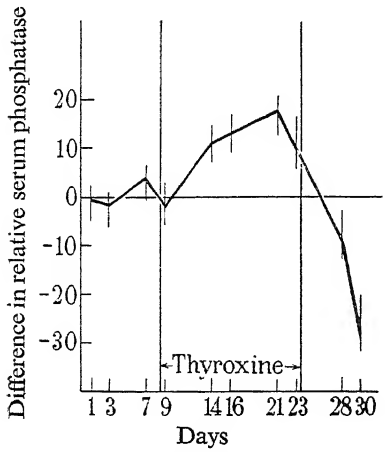

FIG. 2—Difference between the mean relative serum phosphatase values for thyroxin injected and control groups of cows. The whole length of each vertical line represents twice the probable error of the determination plotted at its mid-point.

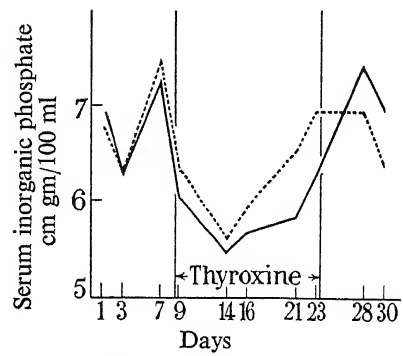

FIG. 3—Mean concentration (mg/100 ml) of inorganic phosphate in the blood sera of thyroxine injected and control groups of cows. —— injected; – – – controls.

Serum Inorganic Phosphate—The group mean values for each occasion of blood sampling are plotted in fig. 3. In calculating the mean values for the control group, the values for one of its members were discarded because the initial level of serum inorganic phosphate for the particular cow differed from the group mean by seven times the standard deviation. The diagram shows that thyroxine treatment at the dosage used, caused in the injected cows a hypophosphataemia relative to the control animals. After the injections had ceased the hypophosphataemia quickly disappeared and was replaced by a relative hyperphosphataemia which persisted as long as observations were made.

As between the injection period and the corresponding control period, the injected animals show a mean decrease of 1·02 mg of inorganic

phosphate/100 ml serum (with a standard error of 0·095 mg/100 ml), while the control cows show a mean decrease of 0·59 mg/100 ml (with a standard error of 0·20 mg/100 ml). The net effect of the injections therefore is a fall in serum inorganic phosphate amounting to 0·43 mg/100 ml (with a standard error of 0·20 mg/100 ml), *i.e.*, a percentage fall of about 6. (It should be noted here that the significance level of this estimate of the percentage fall is only about 90% (P = 0·1), a little lower than the customary criterion of 95% (P = 0·05) for satisfactory significance.)

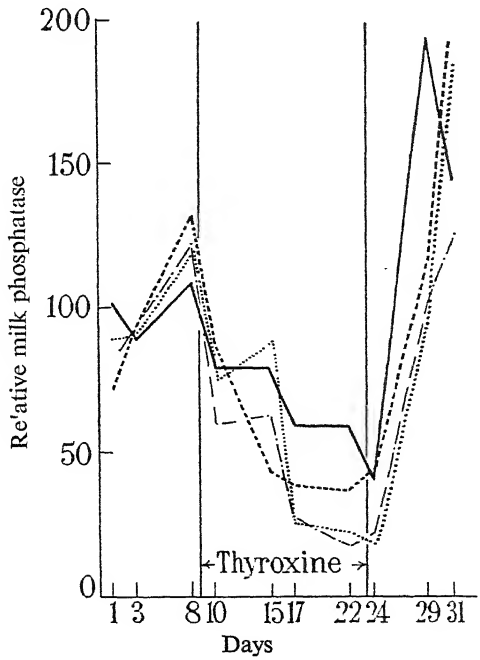

FIG. 4—The effect of thyroxine on the milk phosphatase of four cows. The average initial value for each cow is taken as 100.

Milk Phosphatase—Of all the variables studied, the phosphatase activity of the milk was changed most by the injections of thyroxine. This is immediately apparent in fig. 4, which shows the relative phosphatase activity of the milk of each of the injected animals, and in fig. 5, which shows the mean values for both groups. It is noticeable that the magnitude of the response varies considerably from one animal to another. Further consideration showed that the response was greatest in milks from those cows whose milk normally contains most phosphatase. The relationship between initial level of phosphatase in milk and percentage fall due to thyroxine injections is plotted in fig. 6 and over the range

studied is a remarkably close approximation to the straight line $y = 28 + 0{\cdot}35x$, where y = percentage drop, and x = initial level, measured in Kay and Graham (1933–4) units.

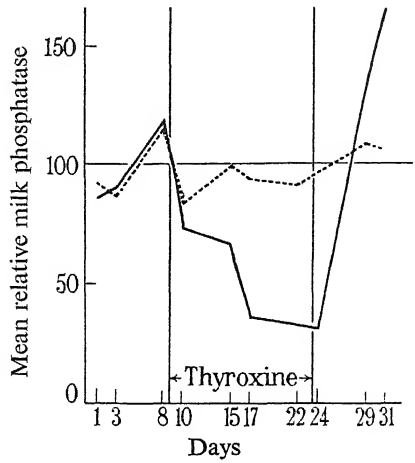

FIG. 5—Mean relative concentration of phosphatase in milk from thyroxine injected and control groups of cows. —— injected; – – – controls.

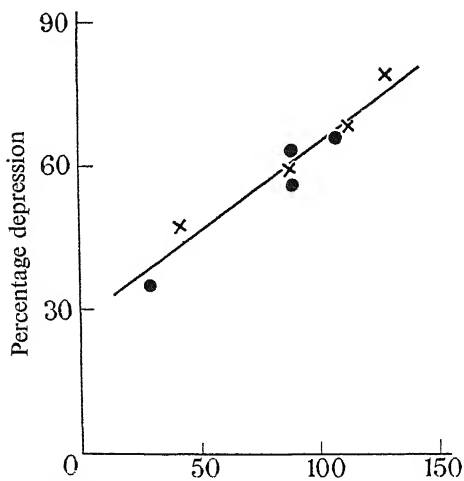

FIG. 6—Relation between initial level of milk phosphatase and percentage decrease due to thyroxine injections. ● morning milk; × evening milk.

A close relationship between initial level of phosphatase in milk and the stage of lactation of the cow concerned was apparent from our results, fig. 7. This accords well with observations previously made in this Institute by Folley and Kay (1936, *b*). By combining this relationship with

the one mentioned above, it is seen that the percentage response of milk phosphatase concentration to thyroxine increases with the progress of lactation.

The depression in the concentration of milk phosphatase resulting from thyroxine treatment persists for two days after the last injection, but on the sixth day therefrom an inverse effect has become established and on the eighth day the magnitude of the after-effect is equal to that of the original response, and no signs of imminent decrease are then apparent.

Since thyroxine injections were accompanied by an increase in milk yield (*see below*) and a concurrent decrease in the concentration of milk phosphatase, the possibility occurred to us that the daily output of phosphatase in the milk remained constant. This is not so, however, since

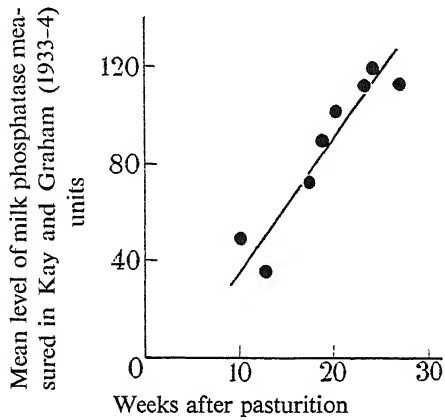

FIG. 7—The relation between concentration of phosphatase in milk and stage of lactation.

in all cases studied by us the fall in milk phosphatase concentration far outweighs the increase in milk yield. Indeed, extrapolation of the straight line in fig. 6 to $x = 0$ shows that the fall in phosphatase concentration predominates over the increase of milk yield at all levels. Thus at $x = 0$, $y = 28\%$, which is the mean percentage increase in milk yield observed in this experiment.

Milk Yield—In fig. 8 are plotted the daily aggregate milk yields of the injected and control groups of cows as well as the daily group yields at each milking. It will be seen that the three lactation curves for the control group of animals fall uniformly. In each case the rate of fall would be best estimated by the slope of the straight line fitted to the curve by the method of least squares. Actually, we approximated to these straight lines by joining the centroids of the first and last eight points in each

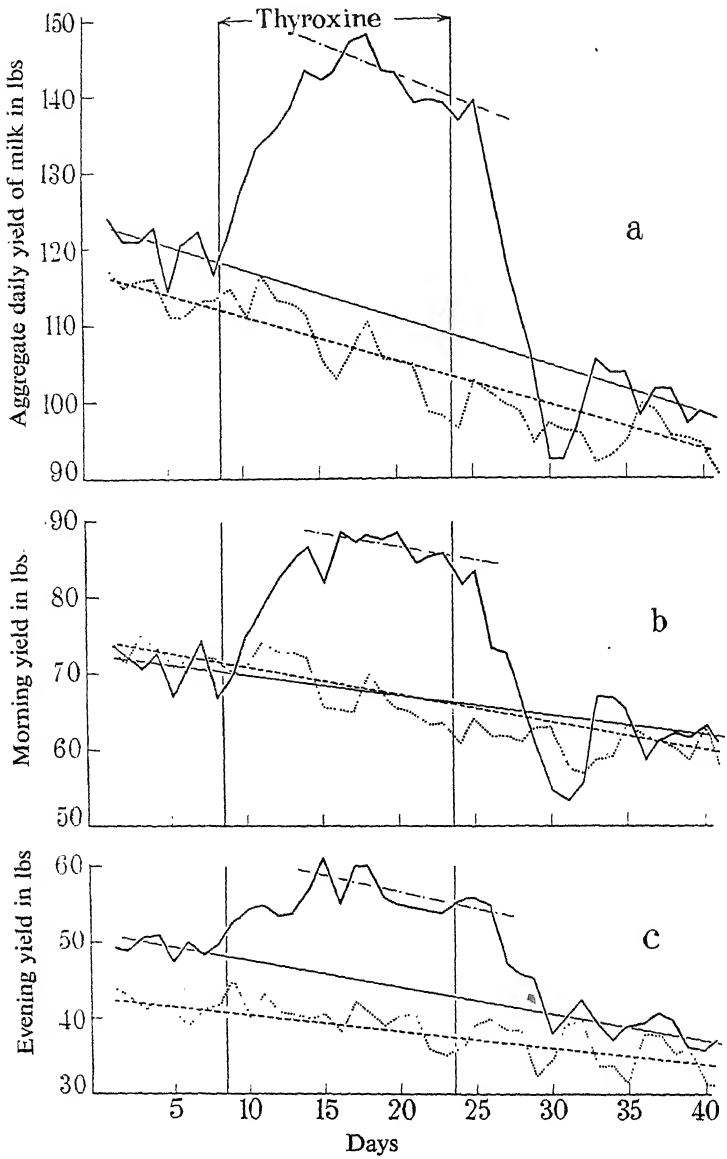

FIG. 8—Aggregate yields of milk from thyroxine injected and control groups of cows. *a*, daily total yield; *b*, yield at morning milking; *c*, yield at evening milking. —— injected; - - - controls; — · — · — · 28% above basal value.

case. This method has the advantage that, since the effects of the hormone were no longer detectable during the last eight days of observations on milk yield, it can be applied also to estimate the probable straight lines which would have fitted the lactation curves of the injected group had no injections been given. The straight lines so obtained can be used to estimate the magnitude of the response to the injections and their ordinates represent conjectural values which we hereafter call the " basal " milk yields.

The great increase in milk yield due to daily injections of 10 mg thyroxine is obvious from the charts in fig. 8. No effect is noticeable at the morning milking immediately after the first injection, but a response is already evident on the evening of that day and its magnitude increases for about seven days. At this stage the milk yields once more begin to decline, but since they do so in a constant ratio to the basal milk yields which are themselves falling, the decline is not to be regarded as a diminution in the effect of the hormone but rather as a manifestation of the natural decrease in yield characteristic of cows in declining lactation.

To examine more closely whether there is any decline superimposed upon that which we ascribe to falling lactation we found, for all three charts, by the method of least squares, the straight lines best fitting the last seven points falling within the injection period and compared their slopes with those of the chain-dotted lines drawn with ordinates 28% above those of the basal lines. Since there was no significant difference it appears as far as can be shown in an experiment involving the injection of four cows only, that the magnitude of the response of milk secretion to the dosage of thyroxine used does not diminish during the first seven days after attaining its full value.

The mean percentage increase in total yield over the basal value taken for the last seven days of the injection period is 28, and the corresponding percentages pertaining to morning and evening milking do not differ significantly from this value.

The response remained constant for two days after the injections were discontinued and then a rapid decline in total daily milk yield set in which brought it back to basal value by the sixth day and below basal for a further period of four days, the greatest deficit below basal being 12%.

Examination of the charts for morning and evening milkings reveals the surprising fact that this negative after-effect occurs solely in the " morning milk ", *i.e.*, the milk secreted during the night. The deficit in morning milk yield attains at its maximum a value of over 16%, while the corresponding evening values agree with the basal values to within

the range of random fluctuations. In addition to this remarkable contrast between the behaviour of morning and evening yields, it is noteworthy that the onset of the decline in yield after cessation of thyroxine

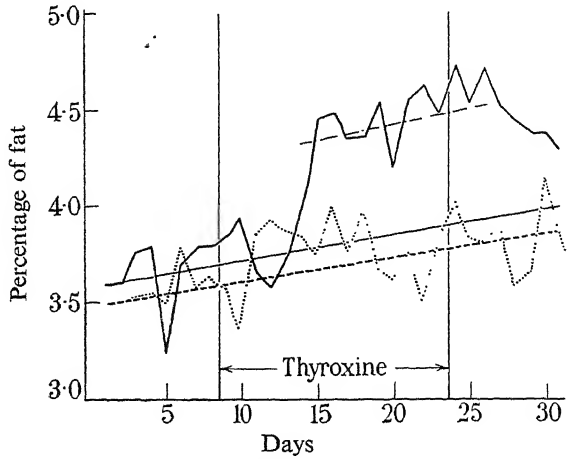

FIG. 9—Mean percentage of fat in milk secreted daily by thyroxine injected and control groups of cows. —— injected; - - - control; —.—.— 16% above basal value.

injections can be detected in the morning milk some 36 hours earlier than the evening milk.

Milk Fat and Milk Non-fatty Solids—In figs. 9 and 10 are plotted the percentages of fat and of non-fatty solids respectively in the total milk

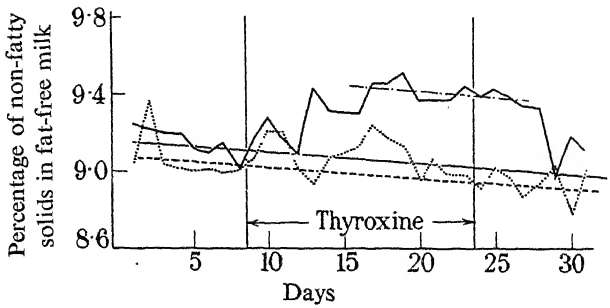

FIG. 10—Mean percentage of non-fatty solids in " fat-free " milk secreted daily by thyroxine injected and control groups of cows. —— injected; - - - control; —.—.— 4% above basal value.

secreted daily by each group. In the curves relating to the control group, the straight lines have been fitted by the technique used in fig. 8. Similar treatment of the curves relating to the injected animals is impossible in this case since the after-effects of the injections have not entirely dis-

appeared by the end of the period of observations on fat and non-fatty solids. A conjectural basal line has therefore been supplied in each figure by drawing through the centroid of the first eight points a straight line parallel to the straight line fitted to the control curve.

Milk Fat—It will be noted that thyroxine injections increase the fat percentage of milk but that the response is much longer delayed than for milk yield. During the first five days of injections no change in the percentage of fat could be observed though the milk yield was rising vigorously. By the eighth day, however, an increase of 16% had been established and remained at this level, within the limits of random fluctuations, until the fourth clear day after the cessation of injections. At this point a decline set in which was more gradual than for milk yield, and which was not complete on the eighth clear day after injections ceased.

TABLE II—RESPONSE OF FAT CONCENTRATION TO THYROXINE
Difference between response in morning and evening milk

Time of day	Concentration of fat in gm/100 ml (mean of last seven days of injection period)		Percentage response
	Basal value	Actual value	
Morning milking (*i.e.*, night secretion)	3·25 (±0·08)	3·95 (±0·06)	21 (±3½)
Evening milking (*i.e.*, day secretion)	4·70 (±0·14)	5·13 (±0·12)	9 (±4)
Whole day's milk	3·82 (±0·06)	4·42 (±0·07)	16 (±2½)

(Ranges in brackets are standard errors of means.)

Thyroxine has a significantly greater effect on the percentage of fat in the morning milk than in the evening milk as the figures in Table II show.

The increase in fat percentage in response to thyroxine injections begins at approximately the same time as the response in the milk yield attains its steady value. The total production of milk fat, which is the product of these quantities, therefore rises continuously from the moment the milk yield begins to respond. The increase in fat production finally reaches after eight days a value which is 50% above the basal value and which is steady within the range of random fluctuations.

Milk Non-fatty Solids—Our experiments demonstrate unequivocally that injections of thyroxine cause an increase in the percentage of non-fatty solids in the milk of animals undergoing them. The effect is

relatively much smaller than for milk fat concentration, but there is a general similarity of behaviour in the two.

There is an initial period of four days during which there is no increase in the concentration of non-fatty solids. Thereafter a positive response is evident, which reaches its full value of 4 ± 1% on the ninth day. It is very difficult to distinguish between the magnitudes of the responses evidenced in morning and evening milks since here, owing to the smaller amount of response, the random fluctuations are relatively more important than in the cases considered above. There are indications, however, that the response may be slightly greater in morning milk.

After injections had ceased the response was undiminished for five days, then declined rapidly and by the eighth day had almost disappeared.

As for total fat production, the total production of milk non-fatty solids increased continuously from the time when the response in milk yield began and finally reached a steady value (within the range of random fluctuations) of 33% above basal.

Discussion

Our experiments have demonstrated that in experimental hyperthyroidism, the serum phosphatase level of the lactating cow undergoes an increase. The degree of increase at the dosage of thyroxine used is, however, small (13%) and there is no evidence of the existence of a negative correlation between percentage increase and initial level. It therefore seems that the relatively large range of the normal for serum phosphatase in the Dairy Shorthorn cow cannot be ascribed to individual variations in thyroid activity. It is just possible, however, that greater increases would have been observed with other levels of thyroxine, and further studies using graded doses are projected.

The existence of an inverse relationship between changes in the serum phosphatase and in the serum calcium and/or inorganic phosphate of dogs fed irradiated ergosterol has been reported by Freeman and Farmer (1935). A similar relationship emerges from our results since, though in our experiments thyroxine administration was unaccompanied by any significant change in serum calcium, some degree of hypophosphataemia was observed. Our findings differ from those of Rosso (1933) who reported a fall in serum calcium during thyroxine treatment in the dog, and of Heymann and Maier (1933) who found that thyroxine administration to children resulted in hyperphosphataemia, but with no accompanying changes in serum calcium. It is possible that the hypophosphataemia observed by us in lactating animals is connected with the increase in milk

secretion which accompanies experimental hyperthyroidism. Indeed, it accords well with the observations of Graham, Jones, and Kay (reported in the preceding paper) who showed that inorganic phosphate is removed from the blood during its passage through the lactating mammary gland.

In striking contrast to the effect on the serum phosphatase was the sharp *reduction* caused by thyroxine treatment in the concentration of phosphatase in the milk. This contrast points to one of two conclusions; either that the phosphatase of the milk does not come from the blood plasma, or else that while the blood plasma *is* the source of the milk phosphatase, thyroxine profoundly modifies the mechanism by which passage from blood plasma to milk occurs. To discriminate between these two possibilities we calculated the correlation coefficients (r) between the ten available pairs of measurements of serum phosphatase and of milk phosphatase for each of the control cows. These values are given in Table III together with the corresponding values of t, P, and z (Fisher, 1934).

TABLE III—CORRELATION BETWEEN PHOSPHATASE LEVELS IN MILK AND IN BLOOD SERUM OF CONTROL COWS

	r	t	P	z
Peach 3	+0·48	1·53	0·17	+0·52
Winsome 2	+0·52	1·74	0·12	+0·58
Portia 3	−0·24	0·70	0·50	−0·24
Flora 16	−0·09	0·25	0·81	−0·09

Mean value of $z = +0{\cdot}19$ (corresponding to $r = +0{\cdot}19$).
Standard error of mean value of z (theoretically) $1/\sqrt{28} = 0{\cdot}19$
(actually) $= 0{\cdot}21$.

It will be seen that there is no significant evidence of correlation since the mean value of z (0·19) is equal to its own standard error. Moreover, on considering the results for different cows, it does not appear that a high level of serum phosphatase is necessarily associated with a high level of milk phosphatase, even when allowance has been made for the increase in concentration of milk phosphatase as lactation advances. Our results therefore do not support the theory that the phosphatase of milk arises from that of the blood plasma by passage through the cells of the mammary gland.

Any theory put forward to explain the origin of the phosphatase of milk must take account of three facts: (*a*) the percentage reduction due to thyroxine is greatest in those milks in which the initial concentration is highest; (*b*) the concentration of phosphatase in the milk rises as lactation

proceeds, *i.e.*, as milk yield declines; and (*c*) the *total daily output* of phosphatase in the milk is always reduced by throxine whatever the initial level.

Evidence is gradually accumulating which points to the key position in milk secretion occupied by phosphoric esters and hence by the phosphatases which catalyse their formation and breakdown. Thus milk contains acid-soluble phosphoric esters, while one of its major constituents is caseinogen, the molecule of which has been shown by Lipmann (1933) to contain phosphoserine. In connexion with another important milk constituent, lactose, the well-known intervention of phosphorylation and dephosphorylation in carbohydrate metabolism, at once comes to mind. By analogy with the work of Verzâr and Laszt (1934), which suggests that phosphorylation is an intermediate stage in the absorption of fat from the intestine, it is not unlikely that phosphatases are concerned in the production of the third major milk constituent, namely, milk fat.

Evidently the assumption that phosphatases form an essential link in the mechanism of milk secretion is not unjustified and indeed it receives further support from the observations of Gowan and Tobey (1931), who showed that milk secretion is inhibited by phloridzin, which is known to retard phosphatolysis (Lundsgaard, 1933), and from the work of Folley and Kay (1935), who find that mammary gland tissue is strikingly rich in phosphatase.

It is possible that, in order to maintain milk secretion, the mammary gland is continually synthesizing phosphatase somewhat in excess of its requirements for this purpose, the excess being excreted in the milk. Conceivably, as the milk yield declines with advancing lactation, less and less enzyme is necessary for the maintenance of milk secretion and if the rate of enzyme production is maintained or, as is more likely, declines more slowly than the milk yield, the excess excreted in the milk would increase. This would explain the increase in concentration of phosphatase in the milk during the decline of lactation. On this theory thyroxine, by virtue of its action on milk secretion, would bring about conditions which increase the proportion of enzyme required for the purposes of lactation and thus lead to a diminished concentration of the enzyme in the milk. This working hypothesis is admittedly speculative, but in the present state of knowledge regarding the mechanism of milk secretion, it is impossible to advance a more precise theory. A better hypothesis must wait upon further experimental work.

In agreement with those of Graham (1934, *b*), our results demonstrate clearly that injections of thyroxine into cows in declining lactation cause considerable increases in milk secretion and total fat production. In

addition, they show that the total production of milk non-fatty solids is raised by thyroxine treatment (*see* footnote on p. 348). It seems clear that the action of the hormone is to raise the plane of milk secretion, since after attainment of the maximum effect during the injection of thyroxine at a given daily dosage, the milk yield declines at the higher level and at the same rate as if no thyroxine had been administered.

We have further found that the production of milk fat, and milk non-fatty solids continues to increase after the effect of the hormone on milk secretion has reached its maximum, thereby raising the *percentages* of fat and non-fatty solids. This last finding is of particular interest from the point of view of the investigation of the causes underlying the secretion of milk deficient in non-fatty solids—a problem of increasing concern to the agriculturalist at the present time. Further work on the mode of action of thyroxine on milk secretion may yield useful results in this direction.

In our experiments there is clear evidence that following cessation of thyroxine injections, after-effects in the reverse direction from the original responses are established for morning milk yield and concentrations of milk phosphatase, serum inorganic phosphate, and serum phosphatase. There is some delay in the appearance of these negative reactions since the original responses themselves persist for some days after the last injection. These curious effects may possibly be explained as follows: owing to the artificially enhanced concentration of thyroxine circulating in the blood during injections, the production of the hormone by the thyroid gland declines, and a mild degree of hypoactivity of the gland cells is induced. When the external supply of hormone is cut off, it would then be some time before the normal potentiality of the gland as regards thyroxine production becomes fully effective once more.

Our results obtained during the period of recovery from thyroxine treatment reveal the existence of factors which, under certain circumstances, may exert an appreciable effect upon milk secretion during the 15-hour period following the evening milking, while having no effect or a scarcely perceptible one upon milk secretion during the 9-hour period following the morning milking.

Thus the inverse after-effect referred to above is apparent only in the morning milk yields (*i.e.*, in the milk secreted during the night) while the decline in milk secretion from the level established by the injections is earlier apparent in the morning milk than in the evening milk.

If it be true that after injections of thyroxine the thyroid gland becomes temporarily hypoactive, it seems that unknown factors at work, either solely or to a predominating extent during the night, so influence milk

secretion as to render it more sensitive to diminished thyroid activity than it is during the day.

There is evidence in Table II that milk-fat secretion is more sensitive to the influence of thyroxine during the night than during the day, though it is possible that part at least of the observed effect is ascribable to the unequal milking intervals (*see* Bartlett, 1933–4). That night itself has an influence upon the secretion of milk fat (and milk) is indicated by the work of Campbell (1932–3) who showed, with cows milked at similar intervals to ours, that the rate of synthesis of milk and milk fat is higher during the night than during the day.

We gratefully acknowledge the valued criticism and advice of Professor H. D. Kay. The cooperation of Captain J. Golding who provided facilities for the determination of milk fat and total solids and of the Dairy Husbandry Department of this Institute is greatly appreciated. Our thanks are also due to the Government Grant Committee of the Royal Society for a grant to one of us (S. J. F.) towards the cost of the apparatus used in this work.

Summary

Daily subcutaneous injections of thyroxine (approximately 0·02 mg/kg) into cows in declining lactation cause marked increases in milk secretion and in production of milk fat and milk non-fatty solids. The treatment also increases the percentage of fat and non-fatty solids in milk.

During thyroxine treatment the serum phosphatase of lactating cows is somewhat increased, but not to an extent sufficient to justify the conclusion that the marked individual differences which exist in serum phosphatase level among dairy cows are due to individual differences in thyroid activity.

Thyroxine administration causes a sharp decline in the concentration of phosphatase in the milk of cows undergoing it. This response is greatest in milk from those cows whose initial level of milk phosphatase is highest.

No evidence could be obtained in support of the theory that the phosphatase of the milk comes from that of the blood serum.

During the period of recovery from thyroxine injections after-effects in the reverse direction from the original responses were observed in morning milk yield (*i.e.*, milk secreted at night), milk phosphatase, blood serum phosphatase, and serum inorganic phosphate.

Differences between the behaviour of morning and evening milk yields, and between that of the fat percentages in morning and evening milk

during the period of recovery from the effects of thyroxine, suggest that milk secretion is sensitive to factors operating either solely or predominantly at night.

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The Kinetics of Muscle Haemoglobin

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The work of Hill (1933) and Theorell (1934) has shown that mammalian muscle haemoglobin has (1) a hyperbolic dissociation curve, (2) a molecular weight of 34,000, (3) no Bohr effect. In these properties therefore it approximates more closely than blood haemoglobin to an ideal " primitive " respiratory pigment and thus constitutes a more suitable compound for kinetic investigation. A comparison of the known properties of muscle and blood haemoglobins is given in Table I.

Table I—Comparison of Muscle Haemoglobin and Blood Haemoglobin

	Mgb	Hb
Gas-binding properties.		
(1) Shape of oxygen dissociation curve	Always hyperbolic	Sigmoid
(2) Oxygen affinity (from oxygen pressure for half saturation)	Very high (3 to 15 times that of Hb)	—
(3) Effect of p_H on oxygen affinity	Very small	Moderate or large
(4) Relative affinity of CO and O_2: ratio of O_2 and CO_2 pressures at equipartition	20	125–600 horse, 500
Spectroscopic properties.		
(5) Position of alpha band, mμ	582 (Theorell, 1934) 580 (Ray and Paff, 1930)	577
(6) CO–O_2 " span ": shift in position of alpha band, mμ	3·1	5–6
Other properties.		
(7) Molecular weight	34,000 (occasionally also 68,000)	68,000
(8) Ease of oxidation to " met " compound	Very easy	Less easy
(9) Iron content and chemical analysis	Indistinguishable	
(10) Solubility.....	Very much greater than Hb	

The present work consists of determinations of the velocities of combination of oxygen and carbon monoxide with muscle haemoglobin and of the velocities of dissociation of the respective saturated compounds.

The results show that, at the ordinary temperature, muscle haemoglobin satisfies the minimum postulates for a "primitive" respiratory pigment, namely:

(*a*) bimolecular combination of gas with reduced pigment;
(*b*) unimolecular dissociation of saturated pigment;
(*c*) mutual independence of gas-binding groups;
(*d*) small p_H effect.

I—Method and Materials

A—*Use of the Micro-photoelectric Kinetic Apparatus*

The times of half completion for the reactions studied varied from 0·0004 sec to 20 sec. To measure such a wide range of reaction rates the micro-photoelectric form of the Hartridge-Roughton apparatus was employed. The method has been described in detail elsewhere (Roughton and Millikan, 1936; Millikan, 1936); its principle may be summarized as follows. The two reacting solutions are stored in Barcroft tubes suitably protected from the air, fig. 1, and when required are drawn into syringes, from which they are forced at a known velocity into a common chamber by means of an electric motor. Here they are thoroughly mixed and led down an observation tube, at different points of which their degree of reaction is measured with a photoelectric cell colorimeter (Millikan, 1933, *a*). The time it takes for the fluid to go from the mixing chamber to the point of observation is calculated from the measured rate of flow down the tube, and from the distance of the mixing chamber from the point of observation.

The application of photoelectric cell methods to the Hartridge-Roughton apparatus has made possible the use of very narrow observation tubes (1 mm internal diameter) and very dilute solutions, so that the total pigment requirements could be reduced to the quantities of material conveniently obtainable from muscle extracts. About 0·1 gm of pigment (the amount of haemoglobin contained in less than 1 cc of mammalian blood) is now sufficient for a whole series of kinetic curves.

The colorimeter consists of a source of light, two colour filters, a differential photoelectric cell, and a galvanometer. For the present experiments, a suitable combination was found to consist of a mercury arc burner (Vitreosil "point source"), Wratten filters No. 3 and No. 34, a selenium

rectifier type of photocell (SAF, Nüremberg), and a low period, low sensitivity galvanometer (Pye, sensitivity: 1×10^{-8} amp/mm; resistance: 900 ohms; period: 1·5 sec).

The special modifications of technique required for the different reactions are described after the experimental results in each case.

B—*Material*

The muscle haemoglobin was crystallized from aqueous extracts of horse heart as described by Theorell (1932). The band position, measured

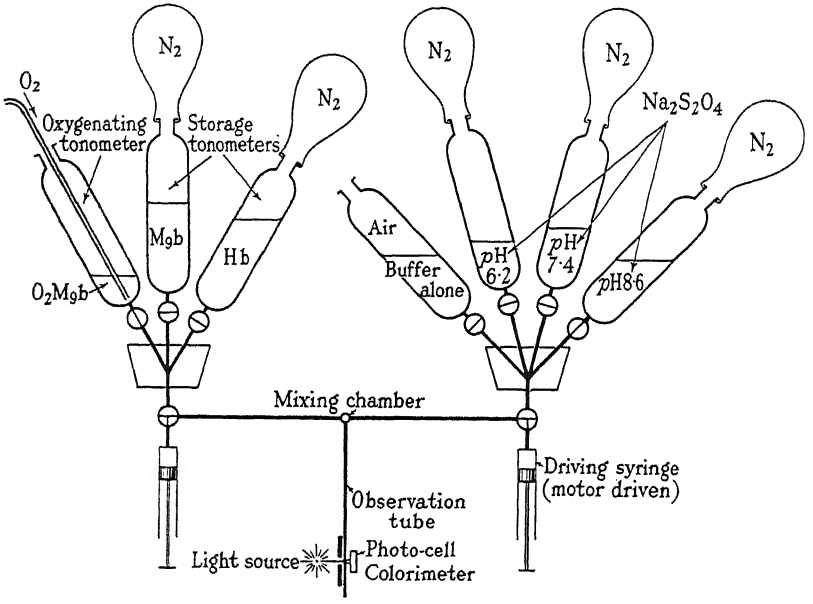


FIG. 1—Diagram of the kinetic apparatus with the solutions used for measuring the dissociation of oxygen from muscle oxyhaemoglobin.

with the reversion spectroscope, was used as a criterion of purity. In some cases the material was recrystallized before use, but no resulting change was observed in its kinetic properties. As a crystal paste, in the presence of ammonium sulphate, muscle haemoglobin will keep for months even at room temperature without apparent deterioration. Owing to its ease of oxidation, it is usually kept as a mixture of " oxy " and " met " muscle haemoglobin, which is converted into the reduced material before use.

C—*Notation and Calculation of Constants*

Since muscle haemoglobin conforms in its gas-binding behaviour to the requirements of a " primitive " pigment, the following simple expressions

may be used in calculating the velocity and equilibrium constants, which are expressed throughout in the units of the papers of Hartridge and Roughton, *i.e.*, time in seconds, and concentrations in millimols per litre.

For O_2	For CO	
K	L	= equilibrium constant
		$= \dfrac{1}{[O_2] \text{ or } [CO] \text{ at } 50\% \text{ satn.}}$ Dimensions: millimols^{-1} (1)
$K = \dfrac{k'}{k}$	$L = \dfrac{l'}{l}$	$= \dfrac{\text{association velocity constant}}{\text{dissociation velocity constant}}$ (2)
k	l	= dissociation velocity constant
		$= \dfrac{\ln 2}{t_{50}} = \dfrac{0 \cdot 7}{t_{50}}$ (3)

where

t_{50} = time of half completion of reaction. Dimensions: sec^{-1}.

k'	l'	= bimolecular association velocity constant (Dimensions: millimols^{-1} sec^{-1})
		$= \dfrac{W}{b \times t_{50}}$, where (4)

b = the concentration of the excess reactant (usually the gas)

t_{50} = time of half completion of the reaction

W = a function of the excess of one reactant over the other, defined by the expression

$$W = \frac{p}{p-1} \ln \frac{2p-1}{p}, \tag{4a}$$

where

$$p = \frac{\text{initial concentration preponderating reactant}}{\text{initial concentration lesser reactant}}.$$

Equation (4) is derived directly from the simple expression for a bimolecular reaction, no account being taken of the back reaction:

$$\frac{dy}{dt} = k'(b-y)\left(\frac{b}{p} - y\right),$$

where

y = concentration of O_2Hb at time t,

b = initial concentration of O_2.

Integrating, we obtain

$$k'(t - t_0) = \frac{1}{b}\frac{p}{p-1}\ln\frac{y-b}{py-b}$$

and substituting for half completion of the reaction, $y = \frac{b}{2p}$ and $t = t_{50}$:

$$k' = \frac{1}{b \times t_{50}}\frac{p}{p-1}\ln\frac{2p-1}{p} = \frac{W}{b \times t_{50}}.$$

The advantage of putting the usual kinetic expression for a bimolecular reaction in the form of equation (4) lies in the fact that b is an easily measured or calculated concentration (O_2 or CO), and W is never very far from 1. If the haemoglobin concentration is not known very accurately, W may still be quite precisely determined, *e.g.*, a 20% error in p, if the latter is about 2, causes only a 1% error in the value of W, and hence only a 1% error in the value of the velocity constant.

II—Experimental Results

Reactions with Oxygen

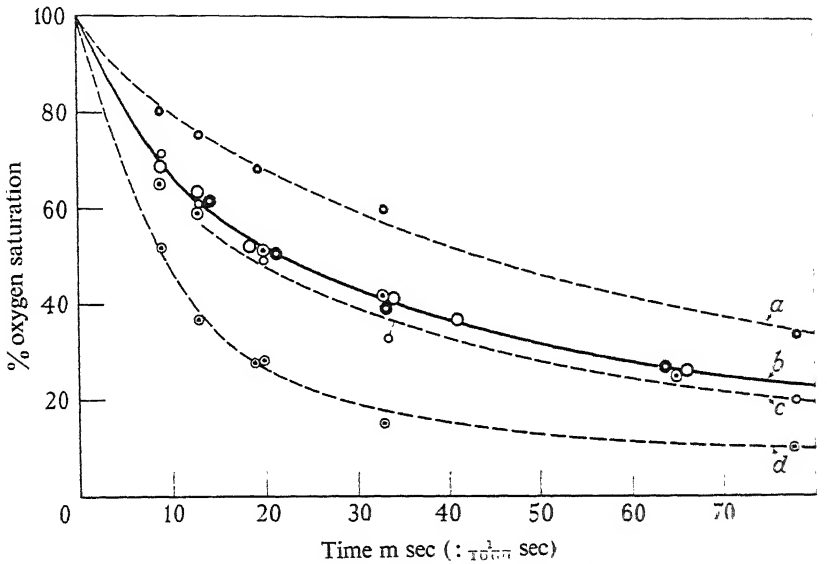
(1) $O_2Mgb - O_2 + Mgb$ in dilute solution.
(Mgb conc. = *ca.* 0·05 gm/100 cc.)

The results of a typical experiment are given in fig. 2. Two points merit attention: (1) the lack of p_H effect, as contrasted with O_2Hb; (2) the identity of the dissociation rate with that of O_2Hb at physiological p_H, the reaction being about half complete in 0·02 sec at 20° C.

Different affinities necessarily reflect differences either in the rate of combination or in the rate of dissociation, or both. Previous experience has shown that it is generally the combination rate which is the stable factor of the two, while the rate of decomposition varies widely (Hartridge and Roughton, 1923, *b*; Millikan, 1933, *b*). The small effect of p_H on the dissociation rate of muscle oxyhaemoglobin is therefore exactly what we should expect in view of the relative insensitivity of its equilibrium curve to p_H changes. This empirical rule has previously been found to hold also for differences between different vertebrate haemoglobins at the same p_H. It is here seen to break down, however, when applied to the blood and muscle pigments of the same animal, for at p_H 7·4, where

the affinities are very different indeed, the dissociation rates are practically equal. We infer, from this fact, a much faster rate of combination for muscle haemoglobin than for haemoglobin.

The dissociation of muscle oxyhaemoglobin is measured by suddenly mixing the oxygenated solution of the pigment with a large excess of sodium hydrosulphite. The oxygen liberated in the decomposition is absorbed before more than a very small fraction of it can recombine, as shown by control tests of the kind described by Hartridge and Roughton



a = Hb p_H 8·6; b = Mgb p_H 6·2, 7·4 and 8·6; c = Hb p_H 7·4; d = Hb p_H 6·2

FIG. 2—The rate of dissociation of O_2Mgb (crystallized material from horse's heart) and O_2Hb (fresh laked human blood) 20° C.

(1925), in which the concentration of $Na_2S_2O_4$ in the observation tube was varied from 0·1 to 0·4 gm per 100 cc.

Because of the great rapidity with which muscle haemoglobin in contact with air goes over into the "met" form, the solution to be used was stored in a tonometer, fig. 1, filled with pure nitrogen. At the start of the experiment this mixture of met-, oxy-, and reduced pigment was all converted into the reduced form by the addition of a small excess of sodium hydrosulphite into the nitrogen-filled tonometer. Before each group of kinetic readings, 5–10 cc were allowed to flow from this storage vessel into another tonometer, through which air was bubbled continuously. Here it was oxygenated in a few seconds, and drawn off into the

driving syringe, before enough time had elapsed for much methaemoglobin to have been formed.

$$(2)\ O_2 + Mgb \rightarrow O_2Mgb$$

The prediction of the last section was confirmed experimentally, for muscle haemoglobin was found to take up oxygen from 2·5 to 5 times faster than blood haemoglobin, fig. 3. This combination of oxygen with

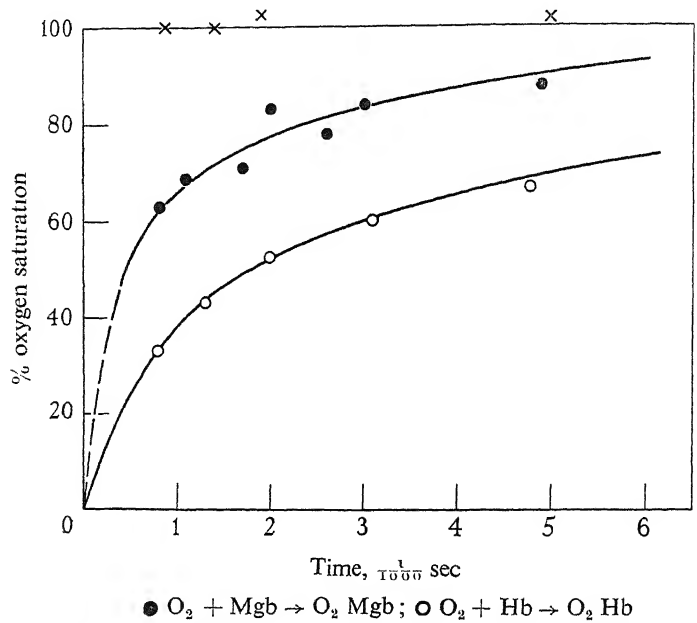


FIG. 3—The rate of combination of oxygen with muscle haemoglobin and with blood haemoglobin, p_H 7·4, 20° C. ● Mgb, total concentration $0{\cdot}62 \times 10^{-4}$ molar; ○ Hb, total concentration $0{\cdot}40 \times 10^{-4}$ molar; oxygen concentration both curves: $1{\cdot}06 \times 10^{-4}$ molar; velocity constants: k' (Mgb) = 19,000 millimols^{-1} × sec^{-1}; k' (Hb) = 4100 millimols^{-1} × sec^{-1}; × × instrumental check: Mgb concentration = same as above; O_2 concentration = six times that above.

muscle haemoglobin is the fastest reaction which has been successfully followed with the kinetic apparatus, and so far as the writer is aware, it is the fastest liquid reaction whose rate has been directly measured. To bring it within the time scale of the apparatus, the concentration of both reactants has to be reduced to a low value, but even with an initial oxygen tension of only 60 mm of Hg, and a muscle haemoglobin concentration equivalent to blood diluted 150 times, the process is found to be more than 60% complete in the first 0·0008 sec. The time for half completion of the reaction under these conditions is about $0{\cdot}0004 \pm 0{\cdot}0001$ sec which is

less than one-fourth of the corresponding time for blood haemoglobin from the same horse. The rates for the blood pigment agree well with the previous results of Hartridge and Roughton for sheep, and of Millikan for human material.

Correlation of Kinetic and Equilibrium Data—The kinetic experiments are of a very different kind from those involved in making the usual oxygen dissociation curves. Therefore, agreement between the values of the equilibrium constant as calculated from the two opposing velocity constants and as obtained directly from a dissociation curve is a very rigorous test of the validity of the method and of the " primitive pigment " assumptions. The experimental values for both oxygen and carbon monoxide have been assembled in Table II.

TABLE II—VELOCITY AND EQUILIBRIUM CONSTANTS FOR MUSCLE HAEMOGLOBIN, O_2, AND CO AT 20° C, p_H 7·4

A. $Mgb + O_2 \underset{k'}{\overset{k}{\rightleftarrows}} MgbO_2$

k'	k	K Calc	K Observed	
19,000 ± 7000	36·6 ± 6·0	520	495	Our experiment
			970	Theorell*

B. $Mgb + CO \underset{l'}{\overset{l}{\rightleftarrows}} MgbCO$

l'	l	L Calc	L Observed	
300 ± 100	0·043 ± 0·01	7000	9500	Our experiment + Theorell's CO/O_2 ratio

* Calculation of equilibrium constant (our units) from Theorell's fig. 4 (1934, *d*, p. 77) is made as follows. His dotted line for 17° C gives log K = 0·45 for p_H 7; *i.e.*, K = 2·80 mm Hg^{-1}. Applying temperature coefficient of Q_{10} = 3·1 (*i.e.*, Q_3 = 1·44) to bring the value to 20° C, we obtain the value for K of 1·94 mm Hg^{-1}, *i.e.*, 970 millimols O_2^{-1}.

The agreement between these values, Table II, is quite as close as the combined experimental uncertainties entitle it to be, when it is remembered that the rate of oxygen uptake is on the very limit of time range of the kinetic apparatus. The discrepancy amounts to 25 in about 500, or 5%. There is, however, a very considerable disagreement between the new values for K and that obtained by Theorell on muscle haemoglobin from the same species and treated in the same way, our affinity values being

about half those found by Theorell. The probable cause of this difference is a concentration effect, for while Theorell's measurements were made on solutions containing 3–6% of haemoglobin, both our kinetic and our equilibrium experiments were made on 0·2% haemoglobin solutions.

The following two control experiments provide evidence that the kinetic apparatus is measuring true reaction velocities and not the speed of mixing of the reactants, even when used for such very rapid processes.

(1) When a reaction which is known to be extremely fast, such as the neutralization of acids by bases is studied in the kinetic apparatus by the thermal method suggested by Hartridge and Roughton (1923, *b*), and developed by Roughton (1930), it was found that 98·5% of the reaction was complete in the first one-thousandth of a second.

(2) By means of a sixfold increase in the initial oxygen concentration in a muscle haemoglobin kinetic experiment, the absolute speed of oxygen uptake was increased to a point where it was more than 95% complete in the first one-thousandth of a second, fig. 3.

Thanks to the kindness of R. Hill, the equilibrium data were obtained with this apparatus and using his Thunberg tube technique. A small amount of dilute haemoglobin solution (concentration, blood diluted 100 times, which is about the concentration used for the kinetic experiments) is completely reduced by evacuation, then successive known amounts of the oxygenated haemoglobin solution are added, the tube being shaken until equilibrium is restored. The oxygen saturation at each oxygen tension is measured inside the tube by a modification of Krogh's optical wedge method, the oxygen tension being calculated from the total amount of oxygen which has been introduced.

Reactions with Carbon Monoxide—The reactions of muscle haemoglobin with CO have less physiological interest than those with O_2, but they are equally important in the elucidation of the physico-chemical properties of the pigment. The difference in the equilibrium behaviour of blood haemoglobin to the two gases was shown by Douglas, Haldane, and Haldane (1912) to be simply one of the parameters involved, and this finding has recently been extended to muscle haemoglobin (Theorell, 1934, *c*). The CO kinetic reactions occur very much more slowly than those involving oxygen, as had previously been shown for blood haemoglobin by Roughton (1934); the increased accuracy obtainable for these slower reactions enables us to examine the *shape* of the kinetic curves more critically than we were justified in doing with the oxygen reactions.

(3) COMgb → CO + Mgb

A typical experiment has been plotted semilogarithmically in fig. 4. The results may be summarized as follows:

(*a*) The reaction follows a strictly unimolecular course throughout the entire measured region which goes down to less than 10% saturation. Furthermore, this character of the dissociation is not affected by an initial acceleration in the rate caused by shining a bright light on the trough for a short time, except that the entire curve is thereby pushed to the left.

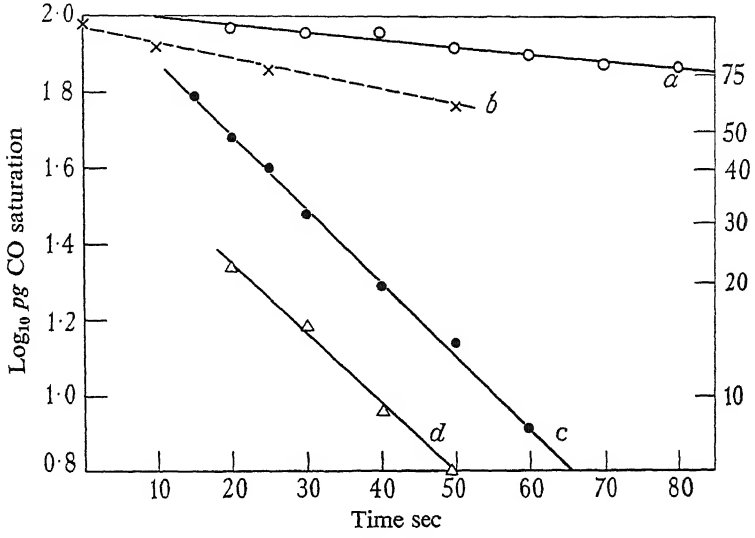


FIG. 4—The rate of dissociation of CO from Hb and Mgb, measured by the ferricyanide method. The ordinates are plotted logarithmically to show the unimolecular form of the curve. The bottom curve shows the effect of photochemical decomposition of COMgb. Temperature 23° C, p_H 6·2 concentration of Hb and Mgb, equivalent to blood diluted 100 times. Tenfold excess of ferricyanide. o Hb, K = 0·0041; × Hb (Roughton, 1934), K = 0·009; ● Mgb, K = 0·045; △ Mgb, with light first 10 sec, K = 0·043.

(*b*) The rate of dissociation of CO is about one-thousandth of that for O_2, although the affinity is only about 20 times as great. This is consistent with the general finding of Roughton (1934) and with the results given in this paper for Hb, from which CO dissociates at a rate about one-tenthousandth of that for O_2, the CO affinity being about 200 times that of O_2.

(*c*) When the muscle pigment is directly compared with that of the blood it is seen that the former loses its CO about ten times as fast as the

latter, although the affinities are very nearly equal. A greater numerical difference between the two substances reveals itself in this reaction than in any other property.*

The reaction was so slow that the Hartridge-Roughton method could be dispensed with and a very simple stationary one substituted in its place. The COMgb was suddenly mixed with ferricyanide, the subsequent disappearance of the COMgb bands being followed qhotoelectrically as the reduced Mgb liberated by dissociation was turned into " met "-Mgb.

Roughton's (1934) data for COHb are included in fig. 4 to show that the present method gives results of the same order of speed as he obtained. The twofold discrepancy between his value for l and ours need not disturb us, inasmuch as the animals used were of different species, and differences of this order have been observed even for different individuals of the same species. The temperatures were within about 2° C of each other.

Critique of the Ferricyanide Method—The measurement of a decomposition usually depends upon immobilizing one or more of the breakdown products, so that the back reaction takes place to a negligible extent. Thus, hydrosulphite has been used to absorb the oxygen liberated by oxyhaemoglobin (Hartridge and Roughton, 1923, and subsequent papers). In the absence of a corresponding reagent for carbon monoxide, Roughton (1934) immobilized the haemoglobin molecule by flooding it with oxygen at such a tension that almost all the " free " molecules were immediately occupied. In the present experiments, we have used ferricyanide to oxidize the haemoglobin to methaemoglobin, special control tests having shown that the direct reaction of ferricyanide with COHb is negligible under the conditions of the experiments.

The reaction was so slow that the streaming fluid method could be dispensed with and a stationary one used in its place. A 1 cm trough of the circular type used by Warburg was about half filled with the carboxyhaemoglobin solution, an equal amount of ferricyanide solution was added and the solutions were mixed in a few seconds by vigorous shaking, one or two glass beads in the trough accelerating the process. The trough was immediately placed in position before the selenium type of photoelectric cell, and galvanometer readings were taken every 5 or 10 seconds, the criterion for the extent of the reaction being the intensity of the alpha and beta bands. The percentage saturation was calculated,

* A much more striking difference has just been reported by Morgan, 'J. Biol. Chem.,' vol. 112, p. 557 (1936), the solubility of Mgb is found to be many thousand times as great as that of Hb in buffer solutions.

using the two endpoints for calibration and assuming the validity of Beer's law, which had been shown in a preliminary experiment to hold sufficiently well over a wide concentration range.

Controls of the Method—In order to establish the validity of the prcedure as a method of measuring the reaction, it must be shown that:

(*a*) ferricyanide oxidizes the reduced haemoglobin so fast that the back reaction (recombination of CO with Hb or Mgb) may be neglected;

(*b*) the direct reaction of ferricyanide with COMgb takes place, if at all, much more slowly than the one we are measuring;

(*c*) the light used for measuring the reaction does not itself affect the rate, because of photochemical decomposition of COMgb.

To test condition (*a*) the direct reaction of ferricyanide with reduced haemoglobin solution of strength equivalent to blood diluted 100 times was investigated in the rapid flow kinetic apparatus. When a fortyfold excess of the oxidizing agent was provided, the reaction was found to be more than 90% complete in 0·01 sec, while with only a fourfold excess it was half complete in 0·015 sec. Using the data of the following section on the rate of CO uptake by Mgb it can be shown that, with the concentrations used, CO offers no serious competition to ferricyanide as an absorber of reduced haemoglobin molecules, for less than one out of a hundred free haemoglobin molecules would react with CO before being oxidized.

Condition (*b*) was tested by altering the concentration of the ferricyanide in a CO dissociation experiment. It was hoped that the situation would be analogous to that of hydrosulphite with oxygen, so that there would be no further change in the rate after a certain ferricyanide concentration had been reached. A slight positive effect was, however, found, a tenfold increase in the ferricyanide resulting in a 60% increase in the rate of oxidation of the pigment. This slight increase may be due to direct action of ferricyanide on COHb, or alternatively to some type of salt action caused by the high concentration of ferricyanide (up to 20%). The small amount of COHb lost by direct attack could very easily be corrected for, by extrapolation to zero ferricyanide concentration, but it was found in practice that the correction was less than the experimental error of the measurements.

It was found that condition (*c*) could be satisfied by making a large reduction in the intensity of the incident light. To compensate for the attendant loss of photoelectric current, the surface illuminated was made about 100 times greater than in the kinetic apparatus, the normal 1 mm observation tube being replaced by a parallel-sided trough several square

centimetres in area. The absence of a "light effect" was shown by the absence of any change in the reaction when the dim light was reduced to one-half. A large increase in the light intensity did, however, accelerate the reaction, as referred to above (*see* fig. 4).

Roughton has raised the objection to the ferricyanide method, as applied to haemoglobin, that we know nothing about the influence of oxidizing one or more of the iron atoms on the dissociation of carbon monoxide from another of the four iron atoms in the molecule. In muscle haemoglobin, however, there are only two iron atoms per molecule, and all our evidence suggests that they act quite independently of each other, so that this objection should certainly apply less to the muscle pigment than to that of blood. It is not maintained, however, that this consideration completely frees the method from all stigma of uncertainty; the absolute values of the rates of the reaction should be accepted with somewhat greater caution than those of the other three reactions.

$$(4)\ CO + Mgb \rightarrow COMgb$$

The results may be summarized as follows:

(*a*) the reaction is quite accurately a bimolecular one, fig. 5, in conformity with Roughton's findings on Hb, as well as with the earlier results of Hartridge and Roughton on oxygen uptake by Hb. The second order for this reaction is the only one consistent with a hyperbolic dissociation curve*;

(*b*) as with haemoglobin, the reaction is very much slower than the corresponding one with oxygen; the ratio of velocity constants is about one to sixty, Table II;

(*c*) nevertheless, the muscle pigment reacts with CO two and a half times as rapidly as does that of the blood, fig. 6.

Correlation of Equilibrium and Kinetic Data for CO—We are now in possession of sufficient data to repeat for CO the same calculation which was made for O_2, namely, the determination of the equilibrium constant from the two opposing velocity constants. The figures are given in the last part of Table II. No direct measurements have been made of the equilibrium between CO and Mgb, but the careful work of Theorell on the competition between O_2 and CO enable it to be calculated from the

* A much more stringent test for the order of the reaction has been given by Roughton (1934), who, working with Hb, showed that the bimolecular velocity constant was unaffected by thirtyfold variations of the concentrations of both reactants.

O_2 equilibrium curve with a good deal of confidence. The discrepancy between the values for the equilibrium constant obtained in these completely different ways is here about 30%, being somewhat greater than the corresponding oxygen discrepancy. It is about equal to the combined experimental uncertainties of the different experiments, and is no greater than was to be expected in view of the unknown accuracy of the ferricyanide method for determining one of the velocity constants.

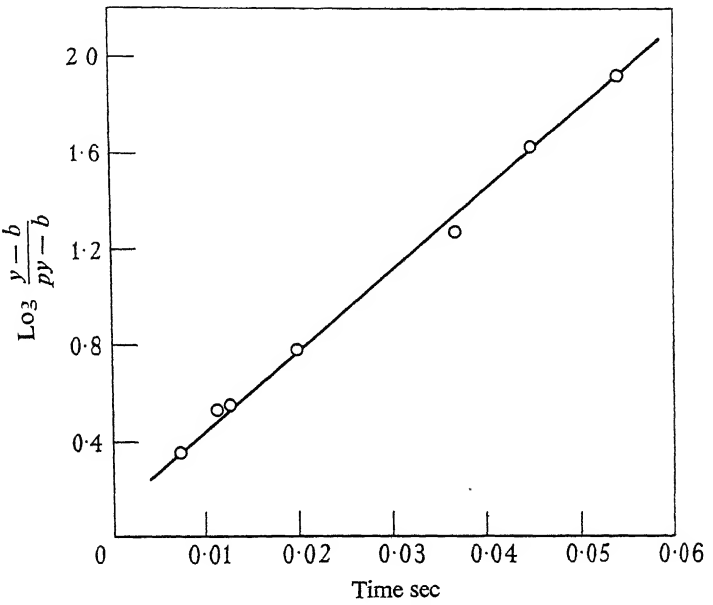


FIG. 5—The order of the reaction

$$CO + Mgb \rightarrow COMgb$$

The ordinates, $ln \frac{y-b}{py-b}$, should give a straight line when plotted against the time, if the reaction is a bimolecular one. Here y is the concentration of COMgb at any time, b is the initial concentration of CO, and p is the initial excess of CO over Mgb.

The Effect of Lead Treatment on the Rate of CO Uptake—Roughly handled haemoglobin solutions have often shown reversion to the "primitive" type in their kinetic and equilibrium behaviour (*see* Roughton, 1934, p. 456). The following control experiment was performed in order to find out whether the lead extraction method, used to remove unwanted muscle proteins, might be responsible for some of the observed differences between dilute laked blood and lead-treated muscle extract.

Four solutions were prepared:

(*a*) muscle haemoglobin, untreated water extract, from horse's heart;
(*b*) the same after lead treatment;
(*c*) laked horse blood from the same horse;
(*d*) the same, after lead treatment.

They were diluted with a buffer at p_H 7·4 until their haemoglobin concentrations were equal, and were then reduced in tonometers by alternate evacuation and flushing with pure nitrogen. They were then used in the same kinetic experiment, the rate of CO uptake being determined because

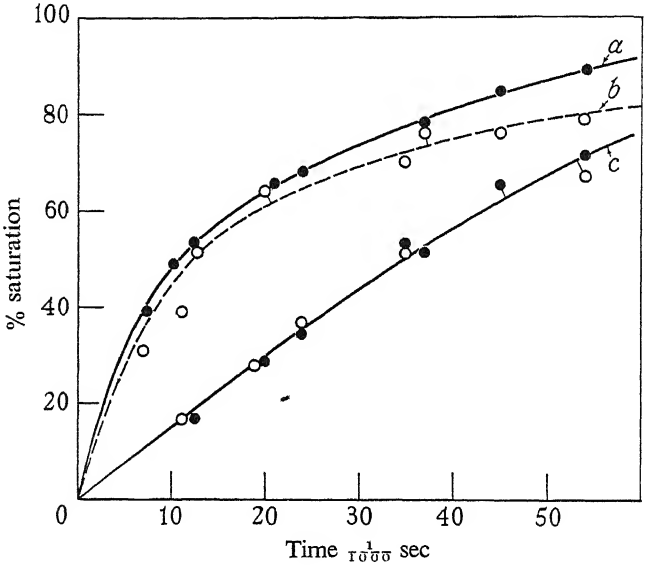


FIG. 6—The rate of CO uptake by Mgb and by Hb, before and after treatment with lead subacetate. p_H 7·4, 20° C. *a* and *b*, CO + Mgb → COMgb; *c*, CO + Hb → COHb; ○, no lead; ●, after precipitation with lead.

it was known to be very different for Mgb and Hb, and because the analogous O_2 reaction is too fast to measure as accurately or conveniently.

The results of the experiment, plotted in fig. 6, prove quite clearly that the handling with lead salts does not cause the blood haemoglobin to approach that of the muscle in its behaviour, nor does it effect a large change in the muscle haemoglobin. The slight increase in rate after removal of the protein impurities is within experimental error in the lower critical portion of the curve, and it is certainly in the direction to be expected if the large amounts of unprecipitated muscle proteins somewhat

" clog " the reaction, either by covering up the pigment molecules or by absorbing the gas ones. The effect only becomes marked near the end of the CO uptake, when any rather slow inhibiting effects might be expected to make themselves felt.

Two previously known facts support the theory that the essential properties are not changed by the extraction process:—

(*a*) The position of the spectral absorption bands is the same in living muscles as in aqueous extracts (Ray and Paff, 1930), and is not altered by the lead treatment.

(*b*) According to some experiments of R. Hill (*unpublished*), the oxygen dissociation curve is little affected by the lead treatment.

Controls—The method used for reaction (4) was identical with that of reaction (2), except that the velocities being less, more leisurely rates of flow down the observation tube could be used (1 to 2·5 metres per sec, instead of 2 to 4·2), and measurements could be taken at a more convenient part of the tube.

Three control factors were considered: (1) the speed of mixing of the reduced pigment with the CO; (2) the occurrence of the back reaction; and (3) the effect of light. The speed of mixing in the apparatus was adequate, because it had already been shown to be sufficient for the much faster oxygen reaction. The back reaction could be neglected, because it had already been measured, and had been found to be very slow indeed. The effect of light was tested in simple control experiments in which the rate of flow, and hence the time of exposure, was varied over a wide range. It was found that the photochemical decomposition of carboxyhaemoglobin is a negligible factor, even at times of exposure much longer than those used in the kinetic experiments.

III—Discussion of Results

(*a*) *Internal Consistency of Results*

We have seen how all four of the reactions measured fit the simple picture presented by the hyperbolic form of the oxygen and carbon monoxide dissociation curves. By choosing a " simple " substance, we have been able to avoid theoretical complications necessitated by S-shaped dissociation curves, and those due to p_H changes. Each reaction, either kinetic or equilibrium, is characterized by a single constant of unequivocal meaning, and these constants are mutually consistent.

(*b*) *Comparison of Muscle Haemoglobin and Haemoglobin*

In considering how the differences between this " primitive " pigment and haemoglobin might be brought about we must analyse these differences as traced back to their kinetic components. The relative rates of reaction of the two pigments have been collected in Table III. The most striking feature here presented is the reduction in velocity of the " on " reaction

TABLE III—RELATIVE SPEEDS OF REACTION

Muscle haemoglobin and blood haemoglobin

(1) $O_2Mgb \rightarrow O_2 + Mgb$. p_H 7·4, 20° C.

Date	Ratio $\frac{k_{Mgb}}{k_{Hb}}$	Mgb (horse heart)	Hb
1934			
September 27	1·03	Crystal (Berlin)	Human
	0·78	The same	Same
1935			
March 2	0·68	Pb ppt	Pb ppt Same horse
March 17	0·48	Pb ppt	Pb ppt Same horse
April 30	0·69	Filtered extract	Laked blood

Averages: $\frac{k_{Mgb,\ horse}}{k_{Hb,\ human}} = 0{\cdot}90$

$\frac{k_{Mgb,\ horse}}{k_{Hb,\ horse}} = 0{\cdot}62$

(2) $O_2 + Mgb \rightarrow O_2Mgb$. 20° C ± 2° C. p_H 7·4.

Date	k'_{Mgb}	k'_{Hb}	Ratio: $\frac{k'_{Mgb}}{k'_{Hb}}$	Mgb (horse heart)	Hb
1934					
August 27	—	—	7	Crystallized (Berlin)	Fresh human
1935					
January 11	—	—	5–6	2*x* crystallized (Cambridge)	Fresh human
January 18	13,500	1700	8	2*x* crystallized	Sheep
February 15.....	13,100	2210	5·9	Muscle extract	Fresh human
February 20.....	—	—	3·3	Muscle extract	Horse
	—	—	5·5		
April 30	19,000	4010	4·5	Pb ppt	Horse

Average, ratio:

$\frac{k'_{horse\ Mgb}}{k'_{human\ Hb}} = 6{\cdot}1$

$\frac{k'_{horse\ Mgb}}{k'_{horse\ Hb}} = 4{\cdot}4$

TABLE III—(continued)

(3) COMgb → CO + Mgb.

Date	1 Mgb	l_{Hb}	$\frac{l_{Mgb}}{l_{Hb}}$	Mgb	Hb
1934					
August 2	0·0465	0·0040	11·6	Crystallized horse heart	Rabbit blood

Ratio, single detmn $\frac{l_{Mgb,\ horse}}{l_{Hb,\ rabbit}} = 11 \cdot 6$

(4) CO + Mgb → COMgb.

Date	$\frac{l'_{Mgb}}{l'_{Hb}}$	Mgb, horse	Hb
1934			
July 31	8–10	Crystal	Rabbit
August 27	13	Crystal	Human
1935			
January 12	9	Crystal	Human
February 22.....	1·4	Pb ppt	Pb ppt, horse
February 23.....	2·2	No Pb	No Pb, horse
February 23.....	2·2	Pb ppt	Pb ppt, horse

Average: $\frac{l'_{Mgb,\ horse}}{l'_{Mb,\ human}} = c.\ 11$

$\frac{l'_{Mgb\ horse}}{l'_{Hb,\ horse}} = 2 \cdot 3$

in passing from the muscle to the blood pigment, it being 2·5 times for CO and 4·5 times for O_2. We are here comparing two reactions whose geometrical configurations can hardly be widely different, the principal distinction being the $n = 2$ molecule for muscle haemoglobin and the $n = 4$ molecule for haemoglobin. Making the simplest assumptions, it can be shown that the " covering " effect caused by this aggregation in the blood pigment, could by itself only reduce the probability of reaction by some 10 to 30%, so that the larger differences actually found are presumably due to a reduction in the size of the " effective target ".

It has been suggested by Adair (private communication) that this reduction of size of the bull's eye in passing from muscle haemoglobin to haemoglobin might allow adjacent targets to get closer together, and hence might account for the mutual interaction of one on another, a condition which is required by Adair's intermediate compound hypothesis in explaining the sigmoid dissociation curves of Hb.

For this purpose some sort of " settling-in " mechanism is usually resorted to, whereby the later oxygen molecules to attach themselves are bound more firmly than they would be if there were no mutual inter-

action between the different oxygen valences. Forbes and Roughton (1931) showed that the last oxygen of all could be made to bear the whole brunt, *i.e.*, only K_4 need be larger than Langmuir predictions, and Pauling (1935) has just shown that the same experimental curves can be obtained on the assumption of a settling-in reaction for each *pair* of " oxyhemes ". In every case the sigmoid curve would lie above the corresponding " primitive " one, *i.e.*, would have a higher affinity as measured by the oxygen tensions at half saturation. Examination of most known dissociation curves, however, shows that exactly the reverse is true, the hyperbolic curves having without exception a higher (50%) affinity than corresponding sigmoid ones. Examples of this empirical rule are Mgb and Hb from the same animal, a reported case of dialysed and undialysed Hb (Barcroft and Roberts, 1909), and the dialysed and undialysed haemocyanins of *Helix*, *Limulus*, and *Busycon*. Similarly the hyperbolic curve of the body fluid of chironomous larva shows a much higher affinity than any of the sigmoid curved vertebrate bloods.

Moreover, " settling in " should reflect itself in a great decrease in the rate of dissociation, and an inconsiderable change in the rate of combination. Actually, the reverse is found to be true. Both the above experimental facts suggest that a reduction in size of the " first oxygen target ", rather than a later " settling in " is desired, and appropriately sigmoid curves can be obtained in this manner. On the present platform of experimental facts, quantitative speculation is perhaps premature.

The rates of dissociation are very differently affected as we pass from muscle haemoglobin to haemoglobin for the two gases. For CO, there is a tenfold slowing up, for O_2 a slight speeding up. This is a reflexion of the much greater *relative* affinity of haemoglobin for CO, but there is no *a priori* reason for expecting such variable behaviour.

(*c*) *Comparison of Haemoglobin, Muscle Haemoglobin, and Haematin Enzyme Systems*

The analogy between the reversible binding of oxygen by blood pigments and of a substrate by its enzyme according to the Michaelis theory has often been commented upon. The geometrical and chemical configurations are in many respects analogous, and a number of corresponding physical chemical properties may be quantitatively compared. Among these are (1) the substrate affinity (reciprocal of Michaelis constant); (2) the velocity of complex formation (not yet directly measured for enzymes, but indirectly calculable as a minimal figure from the specific enzyme activity); (3) the corresponding dissociation velocity constants,

the reaction in one case yielding the original oxygen, in the other the activated or modified substrate; (4) the relative affinity of O_2 and CO for the pigment.

The available data, where applicable, have been collected in Table IV for haemoglobin, muscle haemoglobin, Warburg's oxygen transporting enzyme, and catalase (which is somewhat different since its substrate is

TABLE IV*—COMPARISON OF SOME PHYSICAL CHEMICAL CHARACTERISTICS OF BLOOD HAEMOGLOBIN, MUSCLE HAEMOGLOBIN, CATALASE, AND THE OXYGEN TRANSPORTING ENZYME

(Neutral p_H, room temperature, figures only approximate)

Property	Hb horse	Mgb horse	O_2 transporting enzyme Yeast, coccus, etc.	Catalase Horse liver
Substrate (oxygen) concentration at one-half saturation, 1/millimols	$1 \cdot 7 \times 10^{-2}$	$1 \cdot 5 \times 10^{-3}$	$< 5 \times 10^{-5}$	$2 \cdot 5 \times 10^{-1}$
"On" velocity constant, k', k_1 resp. 1/sec × millimol..	7×10^3	$1 \cdot 7 \times 10^4$	$> 2 \cdot 3 \times 10^6$	$> 7 \cdot 6 \times 10^3$
"Off" velocity constant	*c.* 40	*c.* 40	$>$ *c.* 5	3×10^5
O_2/CO at equipartition	500	18	1/10	—
Oxidation state of Fe	Bivalent, oxidizable	Bivalent very easily oxidizable	Bivalent and trivalent, alternating?	Trivalent

* The data for this table was obtained from the following sources:—Hb affinity: Ferry and Green (1929). Mgb affinity: Theorell (1934, *d*); and this paper. Atfe affinity: Warburg and Kubowitz (1929). Catalase affinity: Williams (1928); quoted by Haldane (1931). Velocity constants: Hb, Mgb, this paper; Atfe, Warburg, and Kubowitz (1929); Catalase, Zeile, and Hellstrom (1930). O_2/CO ratios: Hb, Barcroft (1928); Mgb, Theorel (1934, *c*); Yeast, Warburg (1928).

H_2O_2 instead of O_2). These figures are essentially those which Haldane (1931) used in calculating the molecular statistics of catalase, to which have been added the more recently obtained values for muscle haemoglobin.

It will be seen that in these properties muscle haemoglobin occupies a position intermediate between blood haemoglobin and the "oxygen transporting enzyme" of Warburg, which has not yet been isolated. In

these properties muscle haemoglobin is seen, therefore, to approach more closely to cellular haematin catalysts than any substances which have yet been obtained in the crystalline state.

The combination of oxygen and muscle haemoglobin is the fastest biological reaction which has yet been directly measured to the knowledge of the writer, with the exception of electrical changes accompanying the transmission of a nervous impulse. It does not compete in speed with the picking up of oxygen by the oxygen transporting enzyme, if the assumptions used by Warburg in calculating this quantity from his very carefully controlled experiments are valid, but it approaches and may surpass the corresponding figure for catalase, which was obtained in a more direct manner from the data of Zeile and Hellstrom (1930).

(*d*) *Muscle Haemoglobin as an Intracellular Indicator of Oxygen Tension*

Regardless of its function in the muscle fibre, muscle haemoglobin may now be regarded as an oxygen tension indicator within the living cell, which operates with a time lag of about one-hundredth of a second at room temperature, and a much shorter one at normal body temperatures. For studying the time relations of rapid oxygen changes, its use will therefore be free from the fundamental limiting factor attending all manometric methods, namely, the time of diffusion of oxygen from the gas phase into the cell. It must be assumed, however, until we have succeeded in measuring the oxygen dissociation curve of muscle haemoglobin *in situ*, that its equilibrium and kinetic behaviour is the same inside the cell and in free solution. This assumption has already been discussed, and it was concluded that it was a reasonable one as a first approximation. Even if there is a considerable difference between the affinities of the pigment in the muscle and in our extracts, it would not invalidate conclusions as to relative changes in oxygen tension nor as to the total amount of oxygen bound or split off during metabolic changes.

(*e*) *Muscle Haemoglobin as an Oxygen Reservoir*

R. Hill has pointed out that the high affinity of muscle haemoglobin admirably fits it for the role of an intermediate oxygen carrier or store, acting between the blood and the enzyme systems of the cells. If this is its true function, we are at once faced with the question: " A store for how long, hours, minutes, or fractions of a second ? " A partial answer to this question can be obtained by comparing the oxygen capacity of the

muscle haemoglobin contained in a normal mammalian heart or other red muscle, and its oxygen consumption. As calculated in this way, the amount of oxygen so stored could not last the tissue more than about 10 or 15 seconds in rest or more than 2 or 3 seconds in high activity. This result implies that muscle haemoglobin may tide the muscle over from one contraction to the next. Such a function would involve very quick loading and unloading of oxygen, of which the experiments reported in this paper have shown the pigment to be capable. Conclusive evidence can only be obtained by direct experiments on living muscle, and as these are now under way, a more detailed discussion of these time relations will be postponed.

I should like to thank Dr. H. Theorell for samples of muscle haemoglobin with which some of the measurements were made, and for his most valuable advice. Some of the experiments were made in the Kaiser Wilhelm Institut für Zellphysiologie, Berlin-Dahlem, the majority of them in the Physiological Laboratory, Cambridge.

Summary

The rates of reaction of muscle haemoglobin with oxygen and carbon monoxide have been measured by means of a micro-photoelectric form of the Hartridge-Roughton streaming fluid apparatus. The approximate velocity constants for extracts of horse heart muscle are compared with those for blood haemoglobin from the same animal in the following table:

Reaction	Muscle	Blood	Dimensions
(1) O_2 combination	19,000	4000	millimols^{-1} sec^{-1}
(2) O_2 dissociation	37	40	sec^{-1}
(3) CO combination	300	130	millimols^{-1} sec^{-1}
(4) CO dissociation	0·04	0·004	sec^{-1}

Temperature 20° C, p_H 7·4.

Muscle haemoglobin is a "primitive" pigment, its dissociation curve being hyperbolic; thence the calculation of the equilibrium constant from the two opposing velocity constants can be made with a minimum of assumptions. The equilibrium constant so calculated agrees with that measured directly within about 5% for oxygen and 20% for carbon monoxide; the combined experimental errors are of about this proportion.

The "combination" reactions for both gases are several times as fast for muscle haemoglobin as for blood haemoglobin; the reaction with oxygen is the fastest one involving biological substances which has yet been measured directly. Under the conditions of the experiment it is

half complete in 0·0004 sec. This high rate of combination accounts for the high oxygen affinity of the pigment, since the dissociation rate is little slower than that for blood haemoglobin. For carbon monoxide, however, the speeds in both directions are much higher for the muscle pigment.

The kinetic results indicate that muscle haemoglobin should be available as a naturally occurring intracellular indicator of oxygen tension, with a time lag of less than 1/100 sec. A new tool for studying the time relations of oxygen consumption in muscle is thus made available.

Its oxygen affinity, its concentration in muscle, and its rates of reaction are all such as to fit muscle haemoglobin for the role of an oxygen store which can tide the muscle over from one contraction to the next. No known property, however, precludes the possibility of its acting catalytically within the cell.

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A Comparison of Various Methods of Measuring the Time-Constant of Accommodation of Nerve

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In an earlier paper Hill (1936, *a*) developed the theory of "accommodation" in excitable tissues. He pointed out that the measurement of its speed may be accomplished in a variety of ways. Experimental details of the method of determining the time-constant of "accommodation" (λ) in nerve, by excitation with exponentially rising currents, have been described by Solandt (1936). In the present paper the value of λ obtained by this method is compared (*a*) with that by alternating current excitation; (*b*) with that by "break" excitation at the anode at the end of a constant current pulse; and (*c*) with that by "make" excitation at the cathode at the end of a gap in a constant current. Excitation by linearly rising currents was not performed, because it is obvious that the value obtained for λ would not differ significantly from that obtained with exponentially rising currents.

METHOD

In all cases the sciatic-gastrocnemius preparation of the Hungarian bullfrog (*Rana esculenta*) was used. The experiments were performed during the winter months (November–February). Details of the treatment of the preparations prior to the determinations of λ are stated with the results. The composition of the normal Ringer's solution used was 6·75 gm NaCl, 0·20 gm $CaCl_2$, and 0·15 gm KCl made to 1 litre with distilled water.

The preparations were always mounted in paraffin-block chambers which carried non-polarizable electrodes (calomel half-cells). These electrodes dipped into shallow pools of Ringer's solution in which were mounted wafers of wood, the nerve making contact with the upper edges of these wafers. The wood wafers had always been soaked in the same solution as the preparation.

Muscle twitch, observed on a lever, was taken as index of nerve excitation. With alternating current stimulation a short maintained tetanus was used.

In all the experiments, except those involving excitation by alternating current, thresholds were measured in volts at the source of the exciting current. The current is directly proportional to the voltage, the latter simply being more convenient to measure.

(*a*) *Currents of Exponential Rise*—This method has been described in detail in a preceding paper (Solandt, 1936). It has been used as a basis for the comparison of the other methods of measuring λ, because it has been shown to give consistent and easily obtained results.

(*b*) *Alternating Current*—The current was supplied by a beat-tone oscillator (N.P.L.—Muirhead) which gave a very pure sine wave over the frequency spectrum used. National Physical Laboratory tests showed a 4·5% harmonic content at 100 cycles per second, and much less at

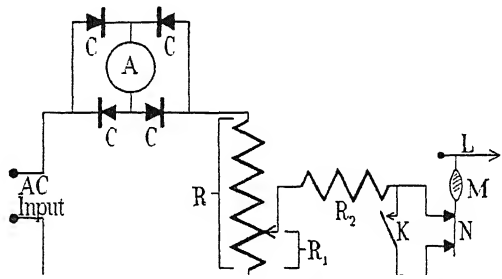

FIG. 1—Circuit for alternating current excitation. A, microammeter; C, copper oxide rectifiers; K, manually operated key; N, nerve on non-polarizable electrodes; M, muscle; and L, lever. Resistance R = 110,000 ohms of which R_1 is variable; R_2 = 952,000 ohms.

higher frequencies, with an additional amplifier not used in the present experiments. Considerably smaller harmonic content would be expected with the oscillator alone. The wave, made visible by a moving coil oscillograph (Downing) and a rotating mirror, showed no detectable deviation from the sinusoidal shape at frequencies above 25 cycles per second. In the present experiments no frequency below 30 cycles per second was used.

The arrangement of the exciting circuit is shown in fig. 1. Non-inductive resistances were used throughout. Care was taken to keep the capacity between the leads from the potential divider to the preparation at a minimum. The current in the output circuit of the oscillator was measured by means of a copper-oxide rectifier unit (four rectifiers connected as shown) and a direct current microammeter. Knowing the value of R_1 the voltage drop across it could be calculated and from this

the current flowing in the nerve circuit determined. The resistance of the nerve and electrodes was measured with a Wheatstone bridge, excited by low frequency alternating current, after each experiment.

The copper oxide rectifier unit had been calibrated previously against a standardized vacuo-junction. The rectifier method of measurement offers the advantages of a linear current scale and the possibility of making a reading as quickly as the meter used permits.

Preparations with a large λ were not suitable for these experiments. With such preparations the optimum frequency n_{op}* is low, and the frequency range below n_{op} is required for determining λ; the wave-form given by the oscillator, as we have mentioned, was not trusted below 25 cycles per second. Moreover, there is danger of repetitive response to each wave, with large values of λ at low frequencies. A preliminary soaking, therefore, of 2 hours in Ringer's solution containing five times the normal $CaCl_2$ was usually given. The preparation was then mounted in a chamber which previously had been brought up to 25° C on an electrically heated sand-bath. By careful regulation of the current through the heater the temperature could be kept constant to within 0·2° C for the duration of an experiment. The high temperature allowed a higher frequency range to be used.

The cut end of the nerve was always very thoroughly injured, by pulping with a pair of forceps, to prevent excitation at the electrode in this region by the positive half-wave. A short maintained tetanus was taken as index of excitation at each cathodal half-wave at the electrode adjacent to the muscle. This was simply observed with the aid of a lever, allowed to write, when desired, on a smoked drum.

(*c*) *Constant Current Pulse*—The circuit shown if fig. 2 was used. Keys A and B were operated by a rotary contact breaker. The interval between the opening of A and of B could be varied from 1·0 to 500 m sec. When A is opened the pulse is initiated as the short circuit across the nerve is removed; the opening of B ends the pulse by breaking the circuit. The timing of the short intervals was checked by inserting a ballistic galvanometer in the circuit and comparing the readings obtained with those given by longer pulses. These results agreed closely, showing that the graduation of the wheel of the rotary contact breaker and the adjustment of the breaker arms were correct.

The potential divider giving fine adjustment of the battery voltage used is not shown in the diagram.

* In this and the paper which will shortly be communicated the optimum frequency is to be denoted, for clearness, n_{op} not n_0.

The electrode near the muscle must be the anode, to give "break" excitation at this point. The cut end of the nerve (cathodal region) was always thoroughly injured with hot paraffin oil, with hot Ringer's solution, or by pulping with forceps. It is very necessary to make this injury

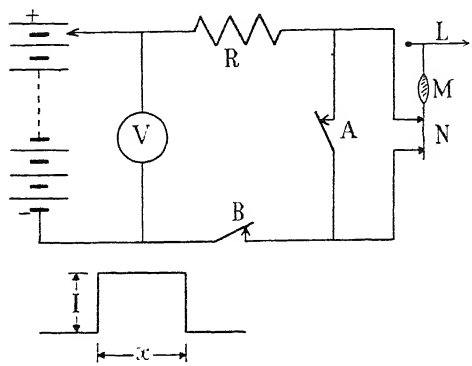

FIG. 2—Circuit for "break" excitation at end of constant current pulse. V, voltmeter; R, resistance of 1 megohm; A and B, keys operated by rotary contact breaker; N, nerve on non-polarizable electrodes; M, muscle; and L, lever. Current form, I, intensity; x, duration of pulse.

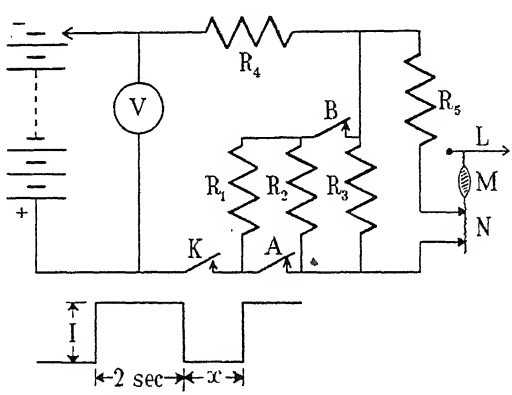

FIG. 3—Circuit for excitation at end of gap in constant current pulse. V, voltmeter; R_1, R_2, R_3, and R_4, resistances each equal to 2000 ohms; $R_5 = \frac{1}{2}$ megohm; K, manually operated key, A and B keys operated by rotary contact breaker. N, nerve on non-polarizable electrodes; M, muscle; and L, lever. Current form, I = intensity; x = duration of gap.

thorough as, with short pulses, "make" excitation at the cathode is difficult to distinguish from the desired "break" excitation.

(*d*) *Gap in a Constant Current Pulse*—The circuit shown in fig. 3, devised by Lucas (1907), was used. Pressing the manually operated key K initiates the pulse, the duration of which was always over 2 seconds.

The keys A and B are opened in this sequence by the same rotary contact breaker as was used for timing the pulses just described. With both keys closed the resistances R_1, R_2, and R_3 are all in parallel. With key A open, key B short-circuits the resistance across which the nerve is connected; this initiates the gap. The opening of B puts the three resistances in series; this ends the gap and gives the stimulating impulse with which we are concerned. The duration of the gap could be varied from 1·0 to 500 m sec.

The strength of current before and after the gap is the same when R_1, R_2, R_3, and R_4 are all equal. In practice a galvanometer was placed in the circuit and R_4 adjusted so that this condition obtained.

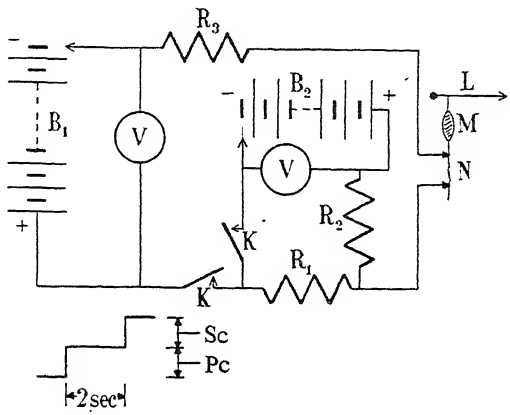

FIG. 4—Circuit for measuring changes of excitability due to catelectrotonus. V, voltmeters; K, manually operated keys; B_1, polarizing battery; and B_2, battery to supply test stimulus. R_1 = 10,000 ohms; R_2 and R_3 each 1 megohm. N, nerve on non-polarizable electrodes; M, muscle; and L, lever. Sc, stimulating current; Pc, polarizing current.

The potential divider giving fine adjustment of the battery voltage used is not shown in the diagram.

The cut end of the nerve (anodal region) was thoroughly injured by pulping with forceps to prevent " break " excitation at the initiation of the gap.

Catelectrotonus was found to be of importance in these experiments. The circuit shown in fig. 4 was used to determine the changes of excitability resulting from it. The nerve was polarized with successively stronger constant currents from B_1. The threshold to the " make " of a constant current test-stimulus from B_2 was found for each polarizing current. The polarizing current was allowed to flow about 2 seconds before the test stimulus was applied.

Preparations were usually tested for catelectrotonic changes of excitability after the determinations of λ had been completed. In many cases soaking in K-rich Ringer's solution was performed to reduce, obliterate, or reverse the normal catelectrotonic effects (Chweitzer, 1935).

Results

(*a*) *Alternating Current*—Table I shows a typical set of frequency-threshold data for alternating current stimulation. The experiment is very simple to perform and the results are consistently repeatable. Care must be taken, however, to take a maintained tetanus and not a twitch as index of excitation. Hill (1936, *a*) has discussed the anomalous twitch response which may result from "making" the current through the nerve at an arbitrary phase in the cycle.

If I is the current through the nerve (fig. 1),

$$I = \frac{iR_1}{R_1 + R_x},$$

where i is the current flowing through the rectifier and R; R_x is the resistance of the preparation and electrodes plus R_2; R_1 is a part of R as indicated.

Table I—Alternating Current Excitation

Frequency n (cycles/sec)	Current i read on meter		R_1 (*see* fig. 1)	
	μA ↓	μA ↑	Ohms ↓	Ohms ↑
150	115	115	190	212
110	114	112	197	210
80	113	111	210	217
65	109	109	219	229
55	109	107	232	240
50	107	107	244	249
45	105	105	257	261
40	102	102	271	278
35	99·5	99·5	300	302
30	95·0	95·0	338	340

Temperature = 25° C.

Hill (1936, *a*) has shown that,

$$\frac{I^2}{I_0^2} = (1 + 4\pi^2k^2n^2)\left(1 + \frac{1}{4\pi^2\lambda^2n^2}\right),$$

where I is the threshold at frequency n, I_0 is the "true" rheobase, k is the time-constant of excitation, and λ is the time-constant of accom-

modation. For the present purpose, *i.e.*, the determination of λ, frequencies lower than the optimum must be used, and the threshold is largely affected by λ; the term involving k may, without significant error, be neglected. Thus,

$$\frac{I^2}{I_0^2} = 1 + \frac{1}{4\pi^2\lambda^2 n^2}.$$

TABLE II—DATA FROM TABLE I TREATED TO GIVE $10^4/n^2$ AND I^2

n cycles/sec	$10^4/n^2$	i (mean) μA	R_1 (mean) Ohms	$R_1 + R_x$ Ohms	I μA	I^2
150	0·45	115	20100	1062 × 10^3	2·18	4·77
110	0·83	113	20350	1062	2·16	4·68
80	1·56	112	21350	1063	2·24	5·01
65	2·36	109	22400	1064	2·30	5·29
55	3·30	108	23600	1066	2·39	5·69
50	4·00	107	24650	1067	2·47	6·09
45	4·93	105	25900	1068	2·54	6·49
40	6·26	102	27450	1069	2·61	6·83
35	8·17	99·5	30100	1072	2·79	7·78
30	11·11	95·0	33900	1076	3·00	8·97

$R_x = R_2$ plus preparation and electrode resistance.

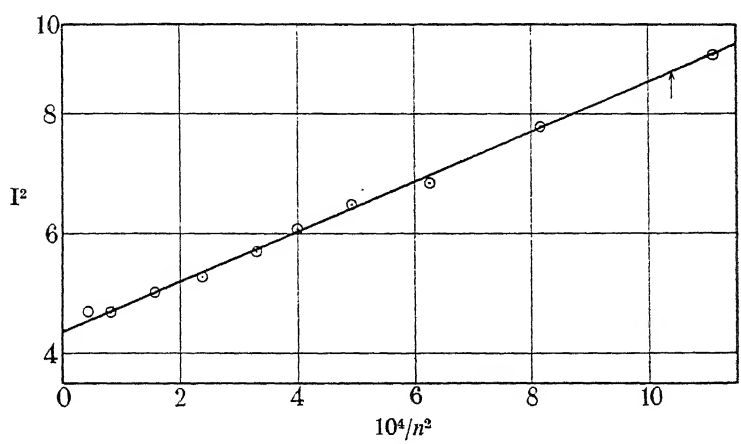

FIG. 5—Excitation by alternating current. I, current threshold for excitation at n cycles per second (drawn from data in Table II). λ = 5·1 m sec; temperature, 25° C. Same preparation as used for experiment of Table III and fig. 6.

We plot I^2 against $1/n^2$. Table II gives these values derived from the data of Table I. Fig. 5 shows that the result is a good straight line. The square of the "true" rheobase (I_0) is given by the intercept of this line on the vertical axis. Let us take a point where $I^2/I_0^2 = 2$ as marked by

the arrow. This corresponds to $1/n^2 = 10 \cdot 4 \times 10^{-4}$. Substituting these values in the above expression for I^2/I_0^2 and transposing we get,

$$\lambda^2 = \frac{10 \cdot 4 \times 10^{-4}}{39 \cdot 5}$$

$$\lambda = 5 \cdot 1 \times 10^{-3} \text{ sec} = 5 \cdot 1 \text{ m sec.}$$

Table III shows data obtained on the same preparation by exciting with exponentially rising currents. In fig. 6 these data are plotted, I/I_1 against the time-constant of current rise (Solandt, 1936). The slope

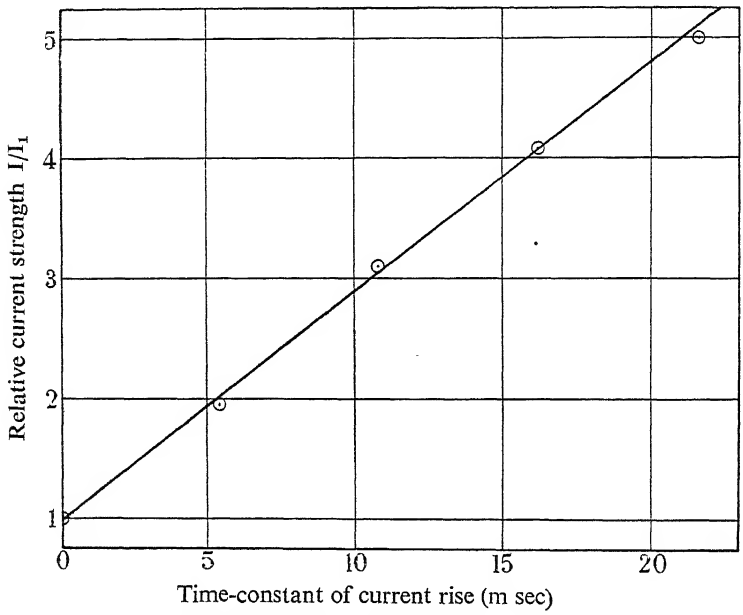

FIG. 6—Excitation by exponentially rising currents. Drawn from data in Table III. Slope of line = 190; $\lambda = 5 \cdot 3$ m sec; temperature, 25° C. Same preparation as used for experiments of Tables I and II and fig. 5.

TABLE III—EXCITATION BY EXPONENTIALLY RISING CURRENTS

Time-constant of current rise (m sec)	I ↓	I ↑	Average of I	$\frac{I}{I_1}$
0	4·0	4·0	$4 \cdot 0 = I_1$	1·00
54	7·7	7·9	7·8	1·95
108	12·2	12·6	12·4	3·10
162	16·2	16·4	16·3	4·07
216	20·0	20·0	20·0	5·00

Temperature = 25° C.

of the resulting straight line is 190. The reciprocal of this slope gives the time-constant of accommodation; in this case $\lambda = 5 \cdot 3$ m sec.

Table IV shows, for a number of preparations, the comparison of λ obtained by alternating current excitation with that obtained by excitation by exponentially rising currents. The agreement is good. All the

TABLE IV—COMPARISON OF λ OBTAINED BY ALTERNATING CURRENT EXCITATION AND BY EXCITATION WITH EXPONENTIALLY RISING CURRENTS

Temperature ° C	Hours soaking in Ringer's solution plus 5 × $CaCl_2$	λ Alternating current excitation m sec	λ Excitation by exponentially rising currents. m sec
24·2	4½	4·4	5·5
24·3	2	8·4	7·5
25·5	2	3·2	4·1
24·3	2	5·3	5·3
24·3	5	5·1	5·2
25·0	5	5·1	5·3
24·5	1	4·6	5·4
24·9	1	4·9	4·9
26·0	4½	2·7	3·4
27·0	4½	3·0	2·8
29·4	2¼	2·6	2·3
27·7	4	4·7	6·7
		Mean = 4·5	Mean = 4·9

values for λ obtained by the latter method have been calculated using the observed rheobase. Hill (1936, *a*) has shown that theoretically more correct values of λ are obtained by making successive approximations to the "true" rheobase and calculating successively "truer" values of λ from these. The "true" rheobase is theoretically lower than the observed value. In the present experiments, however, excitation with repetitive, reversing constant current pulses gave an observed rheobase close to the value of the "true" rheobase. This deviation from theory will be discussed in a later paper on excitation by alternating current. At present it justifies us in using the observed rheobase for calculations of λ.

(*b*) *Constant Current Pulse*—The first two columns of Table V show results obtained for "break" excitation at the anode at the end of a

constant current pulse. There are two methods by which the value of λ may be derived from such data.

Hill (1936, *a*) has shown that

$$I = \frac{I_0 \left(\frac{\lambda}{k}\right)^{\frac{1}{\lambda/k-1}}}{(1-e^{-x/\lambda})^{\frac{1}{1-k/\lambda}}} = \frac{I_1}{(1-e^{-x/\lambda})^{\frac{1}{1-k/\lambda}}},$$

TABLE V—EXCITATION AT THE END OF A CONSTANT CURRENT PULSE

Duration of pulse x m sec	Observed threshold I	I (from smoothed curve drawn from observed values of I)	$\text{Log}_{10}\left(1-\frac{I_1}{I}\right)$ (where $I_1 = 2{\cdot}82$)
40	2·87		
32	2·76		
24	2·82		
16	3·00		
12	3·19		
8	3·69	4·1	$\bar{1}{\cdot}48$
6	5·55	5·1	$\bar{1}{\cdot}64$
4	7·30	7·2	$\bar{1}{\cdot}78$
2	16·0	16·0	$\bar{1}{\cdot}92$

Temperature = 19° C.

where k is the time-constant of excitation, I is the observed threshold on the "break" of a pulse of duration x, I_1 is the "observed" rheobase. With all usual values of k and λ, $1/(1-k/\lambda)$ is approximately unity. The above expression therefore becomes,

$$\frac{I}{I_1} = \frac{1}{1-e^{-x/\lambda}}.$$

Hence,

$$-\log_{10}(1-I_1/I) = (x/\lambda)\log_{10} e,$$

and

$$\lambda = \frac{0{\cdot}434x}{-\log_{10}(1-I_1/I)}.$$

In practice I is plotted against x and a number of values of I and x taken from the smoothed curve. These are given in Table V. Fig. 7 shows the relation between $\log_{10}(1-I_1/I)$ and x in Table V. This relation is best expressed as a straight line. The value of λ is calculated for any point on the straight line. Thus, taking the point in fig. 7 corresponding to 8 m sec on the abscissa we observe that,

$$-\log_{10}(1-I_1/I) = 0{\cdot}52$$

thus

$$\lambda = \frac{8 \times 0{\cdot}434}{0{\cdot}52} = 6{\cdot}7 \text{ m sec.}$$

Another method of deriving λ from the data in Table V is to plot a strength-duration curve in logarithmic coordinates and fit this to the theoretical curve relating $\log_{10} I/I_1$ to $\log_{10} x/\lambda$. This method has been described in detail by Hill (1936, *b*) as a simple way of deriving *k* from strength-duration data for "make" excitation. In our experiments the time-constant involved is λ, not *k*, but Hill has pointed out that the method is equally applicable.

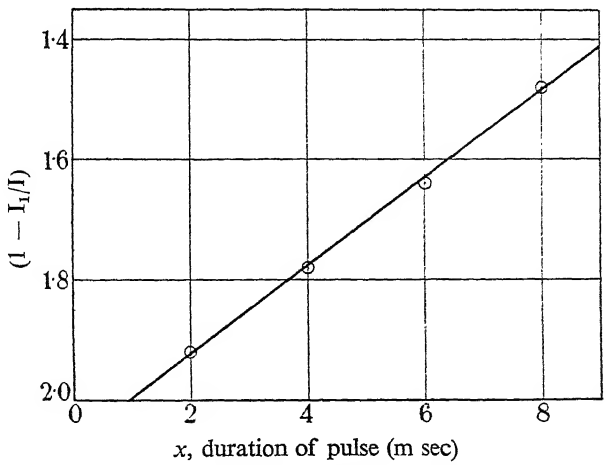

FIG. 7—" Break " excitation at end of constant current pulse. I, current threshold for excitation at pulse duration x; I_1, observed rheobase. Points plotted from data in Table V. $\lambda = 6{\cdot}7$ m sec; temperature, 19° C.

Fig. 8 shows the data of Table V plotted with $\log_{10} x$ as abscissa and $\log_{10} I$ as ordinate. The curve drawn is the theoretical one relating $\log_{10} I/I_0$ to $\log_{10} x/\lambda$. It fits the experimental points fairly closely. The dotted line corresponds to zero abscissa of the theoretical curve. At this point $x = \lambda$. Here $\log_{10} x = 0{\cdot}75$, so $\lambda = 5{\cdot}6$ m sec for this preparation.

The values of λ obtained by the two methods agree well. Usually the latter method has been used because it is simpler.

Table VI shows the comparison, on a number of preparations, of λ obtained by excitation with exponentially rising currents with λ obtained by " break " excitation at the anode as described. The agreement is fairly good.

TABLE VI—COMPARISON BETWEEN λ OBTAINED BY "BREAK" EXCITATION AT THE END OF A CONSTANT CURRENT PULSE AND BY EXCITATION BY EXPONENTIALLY RISING CURRENTS

λ Excitation at the end of a constant current pulse m sec	λ Excitation by exponentially rising currents m sec
6·7	7·9
7·3	7·5
5·4	5·9
6·3	7·9
6·3	8·5
5·6	7·8
5·5	6·3
4·9	7·2
Mean = 6·0	Mean = 7·4

Temperature = 18–20° C.

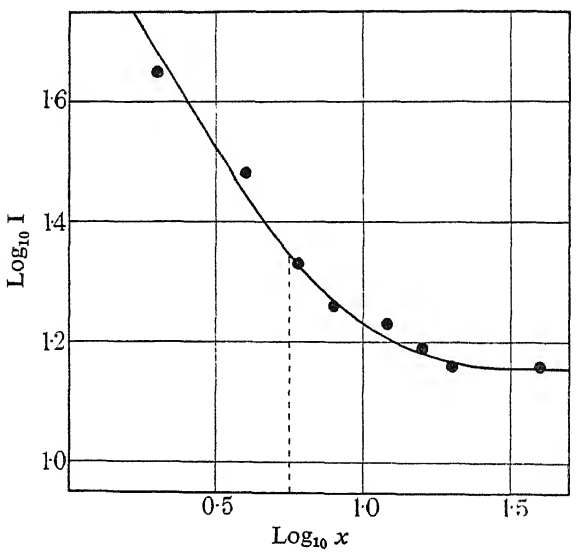

FIG. 8—"Break" excitation at end of constant current pulse. I, current threshold for excitation at pulse duration x. Points plotted from data in Table V. The curve is the theoretical one relating $\log I/I_1$ to $\log x/\lambda$, and the dotted line shows the point where $\log x/\lambda = 0$, or $x/\lambda = 1$. Thus $\lambda = \text{antilog } 0{\cdot}75 = 5{\cdot}6$ m sec; temperature, 19° C.

It will be observed that the values of λ are all low for room temperature (about 18° C). The usual method of injuring the nerve over the cathode, to prevent "make" response, was to cauterize the region with hot oil

or hot Ringer's solution. This always gave a preparation with a low value of λ. It has been observed (Solandt, 1936) that any injured preparation shows a decreasing value of λ. Injuring the cathodal region by pulping the nerve with forceps produced less change in λ. With these preparations, having a value of λ above 10 m sec, the points did not fit the theoretical curve well enough to permit a determination of λ.

(*c*) *Gap in Constant Current Pulse*—Table VII shows data obtained by "make" excitation at the cathode at the end of a gap in a constant current pulse. As the portion of the pulse prior to the gap was long (over 2 seconds) both local potential and threshold had reached final

TABLE VII—EXCITATION AT THE END OF A GAP IN A CONSTANT CURRENT PULSE

Duration of gap x m sec	$\text{Log}_{10} x$	Observed threshold I	Log_{10} I
40	1·60	2·85	0·46
32	1·51	2·85	0·46
24	1·38	2·90	0·46
16	1·20	2·95	0·47
12	1·08	3·40	0·53
8	0·90	3·76	0·58
6	0·78	4·36	0·64
4	0·60	5·60	0·75
2	0·30	10·8	1·03

Temperature = 19° C.

values by the time the gap was initiated. The gap, as Hill (1936, *a*) has pointed out, may be considered as a pulse in the opposite direction. With this picture in mind the excitation at the cathode at the end of the gap becomes excitation at a hypothetical anode at the break of a constant current pulse. As before, λ is the time-constant involved. Here again we can plot the strength-duration (the duration is that of the gap) data in logarithmic coordinates, and obtain λ by fitting the points to the theoretical curve relating $\log_{10} I/I_0$ to $\log_{10} x/\lambda$. Fig. 9 shows the data of Table VII plotted in this manner. The dotted line represents zero on the abscissa of the theoretical curve. Thus $\log_{10} x$, at this point, is 0·78, so λ = 6·0 m sec.

Table VIII shows values of λ obtained in this manner, on a number of preparations, compared with those given by excitation by exponentially rising currents. The agreement is fairly good. All these preparations

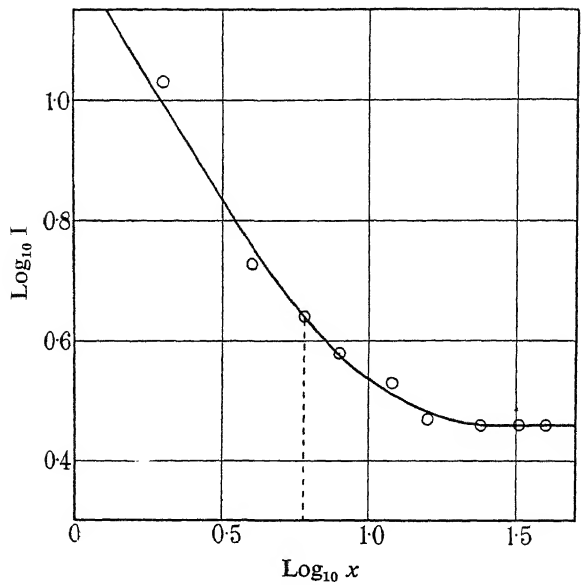

FIG. 9—Excitation at " make ", at end of gap in constant current. I, current threshold for excitation after gap duration x. Points plotted from data in Table VII. The curve is the theoretical one as drawn in fig. 8. $\lambda = 6 \cdot 0$ m sec; temperature, 19° C.

TABLE VIII—COMPARISON OF λ OBTAINED BY EXCITATION AT THE END OF A GAP IN A CONSTANT CURRENT PULSE AND BY EXCITATION WITH EXPONENTIALLY RISING CURRENTS.

λ Excitation at the end of a gap in a constant current pulse m sec	λ Excitation by exponentially rising currents m sec
6·3	6·2
6·3	8·0
6·3	6·3
6·6	6·3
5·8	5·0
4·8	6·8
4·3	5·9
6·0	8·0
Mean = 5·8	Mean = 6·6

Temperature = 18–20° C.

had been soaked in Ringer's solution containing eight times the normal KCl for at least 2 hours.

Soaking in potassium-rich Ringer's solution was first tried because the strength-duration data obtained by this method, after soaking in ordinary Ringer's fluid, did not fit the theoretical curve, and it was thought that catelectrotonic changes in excitability might be the cause. The changes of excitability due to catelectrotonus were tested before and after the determination of λ. Normal catelectrotonus was shown by a lowering of the threshold induced by a polarizing current with the same direction as the stimulating current. Catelectrotonus was regarded as absent when the

TABLE IX—EFFECT OF CATELECTROTONUS ON THE DETERMINATION OF λ BY EXCITATION AT THE END OF A GAP IN A CONSTANT CURRENT PULSE

Catelectrotonus (Maximum lowering of the threshold at the cathode. New threshold expressed as % of the rheobase)	λ by excitation at the end of a gap in a constant current pulse m sec	λ by excitation by exponentially rising currents m sec
Marked (70%)	3·8	8·0
Slight (92%)	4·8	6·8
Marked (60%)	6·3	13·0
None (100%)	4·3	5·9
Marked (50%)	4·0	20·0
Slight (90%)	6·3	6·3

Catelectrotonus was obliterated, partly or wholly, in all the above preparations by soaking in potassium-rich Ringer's solution. Only where the catelectrotonus is nearly or completely absent do the values of λ obtained by the two methods show close agreement. Each pair of preparations was from the same frog. Temperature in every case, 18–20° C.

threshold was unchanged by any polarizing current short of that which itself would excite. In every case the polarizing current was applied for more than 2 seconds before the test stimulating current.

Table IX shows three pairs of preparations, each pair being from the same frog. The preparations were all treated with Ringer's solution containing varying quantities of added KCl. One preparation in each case showed marked catelectrotonus and the other little or no catelectrotonus. In each case the value of λ obtained by the two methods agreed only in the absence of catelectrotonus. Preparations soaked in calcium-rich Ringer's solution have a low value of λ but normal catelectrotonus.

Such preparations give data which fit the theoretical curve poorly. The deduced value of λ, which can only be obtained approximately owing to this poor fit, is much lower than that obtained by excitation by exponentially rising currents.

Discussion

Of the methods cited for measuring λ, that employing excitation by exponentially increasing currents has been taken as our standard of comparison, because it has been shown (Solandt, 1936) to yield consistent results over a large series of experiments. The values of λ obtained by this method, and cited in the present paper, have all been calculated using the observed rheobase. No successive approximation to the theoretical rheobase, as described by Hill (1936, *a*), has been carried out. As previously demonstrated (Solandt, 1936), the deviation from the " true " value of λ which this involves is not great. It is an interesting observation bearing on this point that the " true " rheobase, derived from the alternating current experiments, corresponds very closely to the " observed " rheobase found by excitation with repeated, reversing constant current pulses. The theoretical difference between " true " and " observed " rheobase is not found. The reason for this lies probably in the fall of threshold due to catelectrotonus masking the rise of threshold due to accommodation, as will be discussed in a later paper. In any case the discrepancy probably justifies us, for the present at least, in using the observed rheobase for calculating the value of λ.

The most striking point about the experiments described in this paper is the agreement between the values obtained for λ in excitation by alternating and by exponentially rising currents respectively. The theory (Hill, 1936, *a*), based on the existence of two time-constants of excitation, one being much slower than the other, has made it possible to foretell the changes in threshold which occur when different properties of two quite dissimilar forms of exciting current are varied. In the one case the change in threshold with change in the time-constant of rise of an exponentially increasing current is predicted; in the other the change in threshold with change in frequency of an alternating current is predicted. These predictions have been found to fit the experimental facts, and—fitting them—give us a means of finding the values of the time-constants on which the theory is based. The present experiments have been designed to yield values of λ, and for this reason we have used rates of current rise and frequencies with which the effect of λ on threshold is predominant and that of k negligible.

As Table IV shows the values of λ agree very well although they were arrived at by such different types of excitation. While the difference in the form of the exciting current is striking, the fact that in the one case the excitation is repeated (muscle tetanus taken as index of alternating current stimulation of nerve) and in the other case is single is worth noting. Repeated excitation at these low frequencies apparently produces no change in the time-constant of accommodation of nerve.

The value of λ found with exponentially rising currents is, on the average, slightly larger than that found with alternating currents. Successive approximations to the " true " rheobase would lower the values of λ yielded by excitation by exponentially rising currents. The two values of λ could thus be made to agree more closely. In view of the unexplained experimental agreement between " true " and " observed " rheobase this procedure did not seem justified.

The alternating current method for finding λ depends on the use of an oscillator giving a very pure sine wave. Harmonics which introduce steep places in the rise of the current will produce excitation at a much lower threshold than the frequency justifies. Perhaps this has been the cause of some of the irregular results obtained with alternating current stimulation by earlier workers.

It is only practical to use alternating current for finding λ with nerves having low values of λ. For this reason the preparations used in our experiments were first soaked in calcium-rich Ringer's solution and then the determination made at a fairly high temperature (approximately 25° C). With higher values of λ the optimum frequency becomes lower. In finding λ we must excite with frequencies below the optimum. If muscle response is used as index of nerve excitation at very low frequencies we no longer get a tetanus. It is difficult to tell if a series of twitches is due to one twitch at each cycle, or to repetitive response during a single cycle. This difficulty could be surmounted by amplifying the nerve impulses and watching both them and the exciting current on the screen of a cathode ray tube. There is another difficulty, however, and that is to get a pure sine wave at very low frequencies. Below 20 cycles per second the harmonic content in the output of a valve oscillator is too great. It is necessary to resort to some mechanical (von Kries, 1884; Coppée, 1934) or photoelectric (Nicolai, 1930) method of producing the sinusoidal current. It is really simpler to use excitation by exponentially rising currents for finding λ in all tissues with slow accommodation.

Much work has been done in the past on alternating current stimulation. As none of it deals specifically with the measurement of λ, its mention is left to a later paper in which Hill and his co-workers will discuss in

general the experimental problems of alternating current excitation of nerve.

Cluzet (1908), Cardot and Laugier (1912, *a* and *b*), and Laugier (1921) have shown that a strength-duration curve can be drawn for threshold excitation at the anode at the " break " of a constant current pulse, the duration of the pulse furnishing the time element of the curve. The result is similar in shape to the conventional strength-duration curve obtained by " make " excitation, but the times involved are much longer. The time-constant of the process was found to be ten or more times that of the " make " strength-duration curve. Hill (1936, *a*) has shown that theoretically the time-constant of the " break " strength-duration curve is λ. The data given by Cardot and Laugier (1912, *a*), plotted as a strength-duration curve with logarithmic coordinates, fit the theoretical curve relating $\log I/I_0$ and $\log x/\lambda$ reasonably well, and yield values of λ which are low but within reasonable limits.

The results of the present experiments further confirm the theory. The agreement between λ found by this method and by excitation with exponentially rising currents is quite good when the values of λ are low (below about 10 m sec). With much higher values the data fit the theoretical curve so poorly that it is impossible to deduce a value of λ. The reason for this is not understood.

The fact that a reasonable value for λ can be obtained by excitation at the anode justifies the assumption made by Hill (1936, *a*) that the threshold at the anode undergoes a change in the opposite direction to that at the cathode, but that both have the same time-constant.

Lucas (1907) investigated the change in threshold of excitation at the cathode, at the end of a gap in a constant current, with change in the length of the gap. His data yield rather low values of λ, but these are well within probable limits. He used Ringer's solution with a high calcium-ion concentration which would account for the low values of λ.

Laugier and Dériaud (1924), and Dériaud (1929) performed similar experiments and found that the time-scale of the strength-duration data for this form of " make " excitation was of the same order as that found by Cardot and Laugier (1912, *a*) for " break " excitation. They took the duration of gap which would result in excitation at subsequent " make " at a threshold of twice the rheobase. This time was found to be from 4·6 to 14 times as long as the chronaxie. In only a few cases are sufficient data supplied to draw a strength-duration curve to logarithmic coordinates and to fit to the theoretical curve in the usual manner. Most of the values for λ yielded by this method are very low, in the neighbourhood of 3 m sec. The experiments, however, were mostly performed at a

fairly high temperature (23–26° C), and no account is given of the treatment of the preparations before the determinations were made. Soaking in Ringer's solution rich in calcium or potassium would account for the low values of λ indicated.

The present experiments yielded a good fit to the theoretical curve, and a good agreement with the value of λ given by excitation with exponentially rising currents, under certain conditions. The best agreement was found when the value of λ was below 10 m sec, and when the lowering of threshold associated with catelectrotonus had been abolished by soaking in potassium-rich Ringer's solution. Hill pointed out (1936, *a*, p. 346) that exact quantitative agreement with the theory will often be absent, owing to changes of excitability due to electrotonus. A low value of λ with normal catelectrotonus, as produced by soaking in calcium-rich Ringer's solution, did not result in strength-duration data fitting the theoretical curve. A low value of λ alone is apparently not sufficient to ensure a fit. Lucas (1907) performed his experiments at about 8° C. Eichler (1933) has shown that low temperatures lessen or abolish catelectrotonus. Possibly this is the reason why the data given by Lucas fit the theoretical curve so well.

The author is greatly indebted to Professor A. V. Hill for much essential advice, and for permission to include the results of some of his experiments on the alternating current determination of accommodation. Thanks are due to Mr. A. C. Downing and Mr. J. L. Parkinson for invaluable technical assistance.

Summary

An experimental comparison is made, based on the theory developed by Hill (1936, *a*), of four methods for measuring the time-constant (λ) of accommodation of nerve.

Measurement of λ by excitation with exponentially rising currents was previously investigated (Solandt, 1936). This method is used as a standard of comparison for the other three methods.

Alternating current excitation at frequencies below the optimum yields values of λ which agree well with the standard. This method is easy to use and gives very reproducible results, but is readily applicable only to tissues with small values of λ.

" Break " excitation at the end of a constant current pulse of known duration gives values of λ which agree moderately well with the standard. This agreement only holds when the values of λ are low (below about 10 m sec).

" Make " excitation at the end of a gap of known duration in a constant current pulse yields values of λ which, under certain conditions, agree moderately well with the standard. This agreement is found only when changes of excitability due to electrotonus have been reduced or obliterated by soaking in potassium-rich Ringer's solution.

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Chimpanzee Births in Captivity: a Typical Case History and Report of Sixteen Births

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Twenty years after the announcement by Montané (1915) of the first chimpanzee birth in the New World, which occurred at the estate of the late Señora Rosalia Abreu in Havana, Yerkes (1935) reported the birth of a second-generation captive-born chimpanzee at the Yale Laboratories of Primate Biology, Orange Park, Florida. This was the twelfth of the sixteen births at the laboratories in the last six years. The first case was described by Tinklepaugh (1932), and a twin birth was subsequently announced by Yerkes (1934). The other cases have not been reported previously. Inasmuch as the possibility of breeding chimpanzees successfully in captivity has been demonstrated convincingly in this establishment, it now seems appropriate to describe a typical case of reproduction in the chimpanzee, together with a summary report of all normal births which have occurred here.

The observational data upon which this report is based have been contributed by several individuals. In addition to the writers, those who have contributed most are: William C. Atwater, Kenneth W. Spence, Otto L. Tinklepaugh, Michael I. Tomilin, Ada W. Yerkes, Joseph G. Yoshioka, and S. Zuckerman. We wish to emphasize the importance of collaborative and co-operative endeavour and to acknowledge our indebtedness to our several associates.

Of a score of chimpanzee births reported in Europe and America prior to 1936, few were adequately observed, and only the accounts of von Allesch (1921), Montané (1928), Fox (1929), Tinklepaugh (1932), Wyatt and Vevers (1935), and Schultz and Snyder (1935) offer descriptions of parturitional process. Montané (1915), Blair (1920), Brown (1930), and Clarke (1934) describe pre- and post-parturitional behaviour, but give no description of delivery.

* The assistance of the Committee for Research in Problems of Sex, National Research Council, is acknowledged with appreciation.

In addition to the case (No. 1 in our series) described by Tinklepaugh (1932), two other deliveries have been observed in the Yale colony. Behaviour shortly before and immediately after parturition has been observed and recorded in several instances. Our experience indicates that signs of approaching parturition are difficult to detect without continuous observation. However, our information relative to the gestational period and the events during pregnancy has proved useful in the prediction of the time of delivery, and it enabled us to anticipate and to observe recently the case (No. 13), which has been chosen for detailed description.

The account of mating, gestation, and parturition for the above case is offered as an example of reproduction in the captive chimpanzee. As we have several cases which closely parallel this in the nature and sequence of happenings, we present it as typical. It happens that it is the first case to be reported in which date of conception and duration of prenatal development are accurately known, and one of the few cases in which the description of reproductive process approaches completeness.

The parents of No. 13 (*see* the Table) were female Josie and male Jack. The previous sexual history of the female reads as follows. She arrived at the Yale Laboratories on 10 October, 1930, from the private zoo of Mr. George F. Getz, Holland, Michigan. The hypothetical date of her birth is 1922. Reports from her former owner indicate that she first menstruated in September, 1930. She became pregnant the first time on or about 4 December, 1931, but for undetermined reason this pregnancy terminated in abortion 10 June, 1932. We suspect that either nutritional inadequacy or emotional disturbance was responsible for the mishap. Josie was again mated in October, 1932, and on 15 July, 1933, she gave birth to a normal, full-term infant, which she was allowed to nurse for one year.

Fertilization

We come now to the beginning of the case history to be reported. Menstruation reappeared in Josie on 27 September, 1934, approximately two months after her one-year-old infant had been separated from her. In the course of the next three regular sexual cycles, menstrual bleeding was observed to begin on 7 November, 18 December, and 24 January respectively. Throughout this period Josie was caged with another female and she had no contact with a male until 16 February, 1935, which was the 24th day after the onset of menstrual bleeding, when she was mated with Jack. In this controlled mating and during a thirty-minute period of observation, copulation occurred once. Insemination was

verified by microscopic examination. At the time of mating the sexual skin exhibited maximal swelling. Detumescence began the day following mating.

Pregnancy

Menstrual bleeding did not recur after the mating of 16 February, although a moderate degree of genital swelling appeared for a few days in March and a slight swelling the latter part of May, whereupon the sexual skin returned to and remained in the condition of complete detumescence which we designate as the permanent residual condition. While in this sexual condition, Josie on 30 May was again placed with her consort Jack for a five-minute interval, but he showed no interest in her and there were no sexual contacts.

Although Aschheim-Zondek tests yielded negative results on 29 April and 18 May, interruption of menstruation and changes in Josie's appearance and behaviour indicated pregnancy. By the latter part of June, the abdomen was noticeably enlarged. The Friedman test for pregnancy gave a negative reaction on 3 August. By 31 August vigorous movements of the foetus could be detected by palpation of the abdomen. Descent of the foetus was noted the last week of September, and on 1 and 2 October a white, viscid discharge from the vagina was observed.

Throughout the course of pregnancy Josie was in excellent health and vigorous. A slight decline in weight during the first two months of pregnancy was followed by regular increase from 39·4 kg on 1 May to 46·2 kg on 1 October. Changes in the animal's disposition paralleled these physical events, for she became quieter, more friendly—at times affectionate toward attendants—and increasingly cooperative. Particularly during the last few weeks of the period general activity diminished.

Parturition

Our previous observations suggested that full-term parturition should not be expected in this case before the 230th day of pregnancy, and as we had discovered that brief periods of inspection, although frequently repeated, would not necessarily enable us to predict and observe delivery, we began on 4 October to watch the animal continuously over thirty-minute intervals three times daily. This routine, combined with good fortune, enabled us to observe parturitional process at 6.35 p.m. on 7 October.

The following description of the birth is based upon the observational notes of James H. Elder and William C. Atwater.

The first definite contraction observed occurred at 5.15 p.m. It was followed by another fourteen minutes later. Neither appeared to be very severe and there were no definite signs of distress. Indeed, the event might easily have been overlooked in casual or brief observation. There was irregularity of respiration, with occasional straining and grunting. During the second contraction Josie made an extended sweeping motion with her left leg. She was unusually restless, frequently lying down and rising again almost immediately. The vagina was explored several times with the fingers. There was no enlargement of the vulva; instead, the entire pudendal region seemed contracted and small. As soon as the significance of these signs had become clear, preparations were made for continuous observation. Josie was given two large, clean pieces of burlap, which she at once began to arrange as a nest. She occupied herself in manipulating the cloths for some fifteen minutes before lying upon them.

Although the animal continued to be restless, no additional contractions were observed prior to rupture of the membranes and appearance of the head. Three times in the twenty-five minutes preceding the latter event Josie assumed a squatting posture. It is probable that this behaviour accompanied or facilitated contractions which were not readily observable. The foetal head apparently became engaged before much fluid escaped. At 6.31 Josie assumed a crouching position and a small quantity of fluid was expelled. As she turned to lick it up, excellent opportunity was offered to view the vulva through which the crown of the infant's head was visible. Up to this time no enlargement of the vulva or bulging of the perineum had been noticed. Changes in the female's position, however, prevented continuous observation. Delivery of the head required about two minutes after it was first seen through the vulval opening. Josie at the time lay on her left side, holding the head of the infant with one hand. She did not seem to be aiding or retarding the advance of the trunk, but merely supporting the head. At 6.35 the entire body was delivered in a single contraction. The placenta was expelled three minutes later while the female was in a half-crouching position. She did not pull on the cord. Although the lighting was at times unsatisfactory, observations were sufficiently satisfactory to justify dogmatic statement that this was a case of vertex presentation with occiput anterior. Whether of right or left variety is not known.

Josie at first was more interested in the fluids on the floor of the cage than in her newly born infant, and she busied herself in licking them up. At 6.41 she picked up the infant, which had been left motionless on the floor. When at 6.43 she held it out in front of her, as if for visual inspec-

tion, it cried. Thereupon she put it down, momentarily licked the afterbirth, and then took the baby up again. When it cried she hugged it to her breast and lay on her back.

The next forty-five minutes exhibited monotonous repetition of cuddling the infant, arranging the burlap sacks, licking up fluids from the floor, and chewing at the afterbirth. The baby's cries meanwhile increased in strength and frequency, and correspondingly the mother's attempts to quiet it. The afterbirth had been about half consumed within an hour after delivery, and only a small fragment of it remained in the cage the following day. Loss of blood during and after delivery was relatively slight.

The infant proved to be a strong, active male, whose weight at birth was estimated at about 2 kg. Actual weighing was impracticable because it was not desired to disturb seriously either mother or infant. Within a few minutes after birth the youngster was strongly holding to his mother's hair. From the first he was able to cling to her without assistance. His skin was a very dark brown, and although on crown of head, back, and portions of his limbs there was a fairly abundant coat of jet black hair, the ventral surface was nearly naked.

Puerperium

Forty-eight hours after parturition Josie appeared to be in excellent condition. No symptoms of fatigue or exhaustion were noted at any time. Haemorrhage was not seen after the evening of birth except as noted below. The infant made attempts to nurse within twenty-four hours after delivery, and within seventy-two hours it appeared to obtain an adequate supply of milk.

As particularly important, especially as it might be confused with menstruation, we report that on 22 October a bloody vaginal discharge was observed in Josie. It continued for about forty-eight hours. When first seen this discharge was dark red and contained some mucus. Later, as the flow subsided, it became almost transparent and quite thin. Slight bleeding was observed again the morning of 28 October. We have no explanation to offer for this type of post-partum haemorrhage, but its occurrence is not infrequent.

Summary of essential information concerning fifteen parturitional cases is presented in tabular form. This includes the case which we have described in detail. As there were fourteen single births and one twin birth, the number of infants reported on is sixteen. Following the name of the individual chimpanzee in the Table we have indicated in brackets its

RECORDS OF FIFTEEN CASES OF PARTURITION AND SIXTEEN CHIMPANZEE BIRTHS AT THE YALE LABORATORIES OF PRIMATE BIOLOGY

Case No.	Father	Mother	No. of previous pregnancies	No. of miscarriages	Estimated age at delivery years	Estimated date of conception	Date of parturition	Time of birth	Gestational period days	Infant
1	Pan (3)	Dwina (2)	0	0	10	8 Jan., 1930	11 Sept., 1930	12.14 p.m.	246	Alpha (28)
2	Jack (17)	Mona (36)	2	0	18	Not before 12 Feb., 1931	27 Oct., 1931	2.30 p.m.	Not over 257	Mon (19)
3	Jack (17)	Fifi (38)	1	0	13	Not before 29 Mar., 1931	31 Oct., 1931	Night	Not over 216	Beta (52)
4	Bill (1)	Nana (6)	0	0	11	13 Feb., 1932	21 Sept., 1932	5.00 a.m.	221	Gamma (58)
5	Jack (17)	Pati (42)	2	1	13	8 Sept., 1932	14 May, 1933	Night	248	Ben (23)
6	Pan (3)	Mona (36)	4	1	20	22 Nov., 1932	26 June, 1933	Night	216	Tom (33) Helene (62)
7	Bill (1)	Wendy (4)	0	0	10	14 Nov., 1932	27 June, 1933	Night	225	Bob (35)
8	Bill (1)	Josie (30)	1	1	11	14 Nov., 1932	15 July, 1933	10.30 a.m.	243	Dick (37)
9	Jack (17)	Fifi (38)	2	0	15	19 Apr., 1933	21 Nov., 1933	Night	216	Delta (66)
10	Jack (17)	Dita (40)	2	1	14	3 Aug., 1933	21 Apr., 1934	4.25 p.m.	261	Rosy (68)
11	Pan (3)	Nana (6)	1	0	13	9 Sept., 1933	2 May, 1934	8.00 a.m.	235	Don (39)
12	Bokar (5)	Cuba (46)	0	0	9 y. 18 d.	9 Aug., 1934	11 Apr., 1935	3.00 p.m.	245	Peter (41)
13	Jack (17)	Josie (30)	2	1	13	16 Feb., 1935	7 Oct., 1935	6.35 p.m.	233	Hal (43)
14	Bokar (5)	Wendy (4)	1	0	13	28 June, 1935	16 Jan., 1936	5.15 p.m.	202	Tim (45)
15	Jack (17)	May (32)	0	0	11	17 June, 1935	27 Jan., 1936	Night	224	Lu (72)

number in the laboratory files. The numbers of previous pregnancies and miscarriages are based upon the data in our records. Probably the statements are inaccurate for individuals Fifi, Dita, Mona, and Pati, who had bred prior to their arrival in these laboratories. The ages of parents are hypothetical except for Cuba, whose actual date of birth is known.

Our estimate of the date of conception is based on the assumption that ovulation occurs near the middle of the sexual cycle. Daily records of the sexual status of mature females, which have been kept systematically over several years, show that the mid-point of the cycle almost always falls within the period of maximal genital swelling. The length of cycle which we have used as a criterion of estimate is the median (which in several instances is also the mode) of all recorded cycle lengths for a given individual. For example, the actual length of fifteen sexual cycles in Mona ranges from 30 to 39 days, with a median of 35 days. The mid-point of her cycle is the 18th day. The above method of estimating the date of conception is the best which we have been able to develop in the absence of suitable methods for the direct observation of ovulation. We have used it with full knowledge of uncertainties and probable inaccuracies, primarily because all of the cases in which we have been able to determine the date of ovulation by the method of controlled matings definitely indicate that it occurred at the mid-point for those individuals which show the typical 35-day cycle and after the mid-point in females with longer cycles. In parturitional case No. 13, as described above, the cycles are above average length, with a median and mode of 41 days, and because of the control of mating it is known that ovulation must have occurred between the 23rd and the 25th days of the sexual cycle, or about three days after the mid-point.

The gestational period, as given in the Table, is the number of days from the estimated date of fertilization to but not including the date of parturition. There are two exceptions to this statement, for in cases Nos. 2 and 3 our estimates are necessarily based upon the earliest date of contact with a male, because daily records of sexual status are not available. It is important to note that, for comparison of these determinations with the period of pregnancy in man, where the time usually is reckoned from the onset of the last menstrual period, it is necessary to add to our estimates for chimpanzee the number of days between the beginning of the last menstrual bleeding and the date of ovulation and fertilization. In parturitional case No. 13 of the Table, the estimated gestational period should therefore be increased from 233 to 256 days.

The fifteen gestational periods of chimpanzee which we present exhibit a surprisingly wide range, from 202 to 261 days. Probably this is greater

than occurs in normal full-term cases. In our series the shortest period would be 216 days instead of 202 were we to eliminate from consideration case No. 14, in which we have excellent evidence of prematurity by at least one month. Our determinations, with the exceptions noted, are based upon continuous and immediately recorded observations and are considered to be correct within one to six days. In several of the cases attempt was made to verify pregnancy as early as possible by use of the Aschheim-Zondek test. Some of the results of these tests have been reported by Zuckerman (1935). As far as we may judge at present, the reliability of the test for chimpanzee appears to be rather low.

Attention to the few abnormalities appearing in the fifteen pregnancies under discussion suggests that under appropriate dietary, social, and hygienic conditions, reproduction in captive chimpanzees may proceed naturally and in general uneventfully. Our series of cases exhibits only two fatalities. The mother in case No. 1 died of puerperal sepsis fifteen days after parturition. Prior to that event she had become heavily infested with *Balantidium coli* and was very greatly weakened. Undoubtedly she succumbed to infection following parturition primarily because of her weakness. A detailed account of the case has been published by Tinklepaugh (1932). The second death was that of infant Mon, case No. 2, who died about twenty-four hours after birth, presumably from head injuries either at birth or thereafter. In cases Nos. 1 and 12 the mothers failed to accept and care for their infants properly and they were therefore removed and reared on the bottle. In case No. 1 the pathological condition of the female accounts satisfactorily for the inadequacy of maternal behaviour. The primiparous mother in case No. 12, although normal in all respects before and after parturitional process, apparently was puzzled by the presence of the infant and treated it, as she might any other unfamiliar object, with evidence of curiosity but wholly inappropriately. With rare exceptions, the physical condition of our breeding chimpanzees has been excellent throughout pregnancy and the period of nursing.

Evidence from most of our cases indicates that labour usually is of relatively short duration and that delivery may occur within an hour after observation has failed to reveal premonitory signs. The time apparently may be as short as thirty minutes, and we have no cases of prolonged, difficult, or otherwise abnormal labour to report. It is to be noted that Montané (1915) reports a case lasting only thirty minutes. Fox (1929) described one in which uterine contractions continued for an estimated period of two hours and twenty-five minutes. Tinklepaugh (1932) first noted signs of labour about three hours before delivery, and

in the case observed by Wyatt and Vevers (1935) the period was five hours.

From our records and the literature, it is safe to say that the afterbirth is expelled shortly after the foetus. Evidence to this effect is supplied by Fox (1929), Tinklepaugh (1932), Schultz and Snyder (1935), and at least six of our previously unreported cases. Wyatt and Vevers (1935) state that in a case observed by them the placenta was retained for some thirty hours. The case certainly was atypical. Placentophagia occurred in eight of our fifteen cases. This frequency of occurrence is verified in the literature, for of eight presumably normal parturitional cases, in the report of which we find behaviour toward the afterbirth mentioned, there are four instances of placentophagia. Although we may not infer from these observations that the eating of the afterbirth is necessary to appropriate maternal behaviour and lactation, it probably occurs more frequently than not, and we should be surprised if it were not the typical behaviour of the normal, well-environed, free chimpanzee. It is almost unexceptional that the female during and after parturition licks up all fluids which are available and handles and mouths the afterbirth even if she does not partially or wholly consume it. Quite possibly the absence of placentophagia in some of the cases observed by us has been due to some unusual condition of the female or of her environment. Tinklepaugh and Hartman (1930) state that failure of the monkey *M. rhesus* to consume the afterbirth is associated as a rule with some abnormality of reproductive process.

Repeatedly in these laboratories relations between reproductive process in the female chimpanzee and temperamental characteristics have been noted. Changes in affective behaviour are particularly marked in case of the first pregnancy. As a rule the individual becomes markedly quieter, more gentle, and affectionate. Irritability and unfriendliness are replaced by conspicuous gentleness and cooperation. These changes ordinarily persist throughout the reproductive cycle, and they may even survive separation of infant from mother. They have impressed themselves upon us the more strongly because of their positive value in our work, for not infrequently an individual who during adolescence has been characterized by disagreeableness toward human caretakers becomes an agreeable, easily managed subject with the beginning of pregnancy.

We have presented summary statements concerning several aspects of chimpanzee reproduction which are worthy of more detailed consideration, and to which, in the future, special reports from these laboratories will be devoted. We should like now to discuss, in the light of available facts, various errors and misconceptions relative to the character-

istics of reproductive process in chimpanzee which appear more or less commonly in the literature. The mistakes which we have in mind are typified by those concerning type of presentation, duration of gestation, length of the sexual cycle, relation between genital swelling and menstrual bleeding, identification of genital swelling with menstruation, and occurrence of menstruation during pregnancy.

Misinformation and misleading statements about presentation of the chimpanzee foetus are well exemplified by the recent statements of Vaughn (1935), who asserts that occipital presentation is peculiar to man and " that apes are born snout first ". Were the author not referring specifically to a case of chimpanzee birth, we naturally should assume that she intended to use the term " apes " for primates inferior to the anthropoid apes and man. We do not know of acceptable evidence in support of Vaughn's contention. Instead, it appears that whereas presentation in the monkeys may be and presumably usually is " snout first ", in chimpanzee, as in man, it is cranial. We cite in this connexion Fox (1929), Tinklepaugh (1932), Wyatt and Vevers (1935), and two cases observed by us. In all of the above, vertex presentation occurred, with occiput anterior or posterior.

The duration of prenatal development in chimpanzee has been variously estimated or guessed as from five to ten months. We offer the following as examples of statements available in the literature. Clarke (1934) writes: " Although the preconceived idea is that the period of gestation is nine months, I cannot help feeling that it is five months, at any rate all the evidence points to it in this case. The period of amenorrhea, the copulation observations, and the adolescence (watched) of the male parent are all to my mind strong evidence in favour of this hypothesis ". While by contrast Yerkes and Yerkes (1929, p. 262), in summing up the then available evidences, state that: " The period of gestation is commonly thought to be either seven or nine months, and although it must be granted that the evidences are inconclusive, the probability seems strongly in favour of the latter estimate ". In comment on Clarke's surmise we report that an accurately dated chimpanzee foetus of 186 days, although, except for prematurity, born under favourable conditions, survived for only a few hours. An infant born following a pregnancy of 202 days readily survived. Our experience justifies the assertion that at five months (whether lunar or calendar) of age a chimpanzee foetus, under ordinary conditions, would have not the slightest chance of survival. It appears that heretofore the gestational period has been guessed at instead of observed. We have endeavoured to present certain data which are reasonably reliable, since they are based upon continuous observation.

The average gestational period for our fifteen parturitional cases is 233 days. The median also is 233 days. If cases Nos. 6 and 14, which we consider premature births by not less than one month, be omitted, the average for the thirteen presumptively full-term cases is 236 days; the median, 235. The case recently reported by Wyatt and Vevers (1935) supports our observations, since the gestational period described by them was not less than 239 nor more than 249 days.* Assuming that the modal gestational period for man is 280 less 14 days (the interval between the last menstruation and fertilization), the period for chimpanzee (236 days) is 30 days shorter than that of man (266 days). It may then be asserted, on the basis of our present information, that the gestational periods for chimpanzee and man are about eight and nine calendar months respectively.

As to the length and periodicity of the sexual cycle in chimpanzee, there has existed a superstition that it is approximately the same as in man, or at any rate not longer. Observational basis for statistical statement heretofore has been lacking. There have, it is true, been satisfactory descriptions of a few cycles for isolated individuals. Notable among these are those of Tinklepaugh (1933), Wyatt and Vevers (1935), and Schultz and Snyder (1935). Our continuous systematic records of daily observations for 14 females, including approximately 200 complete cycles, yield an average of between 35 and 36 days. This, a five-week cycle, is comparable with the four-week cycle in man.

A few observers have mistakenly inferred that genital swelling is indicative of menstruation. As a matter of fact, it may or may not be associated with menstrual bleeding. Presumably several of the published mis-statements concerning the duration of gestation are due to this erroneous inference. We suspect that Clarke, as cited above, may have been misled because of unfamiliarity with the characteristics and variability of the sexual cycle. Although it has several times been asserted that menstruation may occur in chimpanzee during pregnancy, we have not observed a single instance. Genital swelling, by contrast, may and frequently does occur, especially early in pregnancy. Almost invariably

* Schultz and Snyder (1935, p. 199), on the basis of data published by Zuckerman (1935, pp. 598–599) from these laboratories, determined the gestational period (" menstrual age ") for seven of the cases in our series. So far as we can tell, their figures in order correspond to our Nos. 5, 8, 6, 7, 9, 10, 11. Their determinations differ from ours because they reckon from the last menstruation, we from the estimated date of conception. For our case No. 11, their figure 241 days appears to be erroneous; it should be 251 days. We present this information to minimize risk of confusion by readers.

it differs from the non-pregnant swelling in that it is less pronounced, of shorter duration, and irregular in occurrence. There is the possibility also that certain observers may have mistaken post-parturitional haemorrhage for menstrual bleeding. Montané (1915), von Allesch (1921), Fox (1929), and Clarke (1934), all refer to the continuation of catamenia during pregnancy. We consider this atypical. Apart from daily observation by an experienced person who is intimately familiar with reproductive process in chimpanzee, the probability of error of observation or of interpretation is so great that we venture to oppose our statements to those of the above authors. It is our surmise that post-parturitional bleeding is the principal source of error and that occasional although rare menstrual bleeding during pregnancy has been accepted as indicative of the persistence and regularity of the process. As a matter of fact, we have depended upon the cessation of menstruation and disappearance or radical modification of the typical genital swelling as signs of pregnancy, although in making tentative diagnosis we have used also other indications, physical, physiological, and behavioural.

Clearly enough, there are many essential features, as well as details, of reproductive process in chimpanzee which require more extended, careful, and critical observation. We venture to suggest as a useful procedure the preparation of a record form as a check list of significant reproductive happenings which should by all means be observed and immediately described. We cannot too strongly emphasize the desirability of continuity and regularity of observation and of general familiarity on the part of the observer with reproductive process as described briefly in this paper, and by Tinklepaugh (1932, 1933).

Summary

This is a report of fifteen pregnancies which terminated in fourteen single births and one twin birth in the chimpanzee breeding colony of Yale Laboratories of Primate Biology at Orange Park, Florida, U.S.A. Following the description of a typical case of reproduction, the authors summarize their findings for the remaining cases. Ordinarily and typically impregnation occurs about or slightly after the mid-point of the 35-day sexual cycle. Menstrual bleeding does not commonly recur after conception. Genital swelling may occur irregularly and in atypical form, especially early in pregnancy.

For thirteen presumptively full-term births the average duration of gestation was 236 days; the median period, 235 days. The extremes for these cases are 216 and 261 days. The period for chimpanzee (from con-

ception to parturition) therefore appears to be about 30 days shorter than in man. Typically the parturient female licks up available amniotic fluid and blood, and frequently either partially or wholly devours the afterbirth. A foetus of 186 days died about seventeen hours after birth; one of 202 days, although very small, survived. Almost without exception the chimpanzee female during pregnancy and nursing is much more gentle, friendly, dependable, and easily handled than at other times.

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The Densities of Protein Crystals and the Hydration of Proteins

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Introduction

The apparent densities of proteins in aqueous solutions have been studied by a number of investigators, including Chick and Martin (1913), and, more recently, by Svedberg and his colleagues, but comparatively few observations of the densities of protein crystals have been published. Eyer (1932) gave the value 1·315 for insulin, Crowfoot (1935) 1·306; Adair and Adair (1934) found 1·296 for haemoglobin; Bernal and Crowfoot (1934) gave 1·28 for pepsin. The media used for the measurements on pepsin and insulin were not recorded.

A method for measuring the densities of small crystals by means of observing their flotation in media of different densities was described by Retgers (1889). The present communication records a modification of his method adapted for the investigation of the density of crystalline and of denatured proteins. The first problem studied was the choice of suitable media. The mixtures of organic liquids which are generally used for density determinations of inorganic crystals are open to objections on the grounds that protein crystals cannot be freed from films of their mother liquor without grave risks of irreversible changes. In working with aqueous media this difficulty need not arise, but the media chosen must fulfil the somewhat exacting requirements of a high density with freedom from the capacity to denature or to dissolve the protein.

It was furthermore necessary to test the validity of the method by measuring the densities of a number of different preparations of a given protein in any one medium. Thirdly, the densities of the protein crystals in different media were compared, and, if different results were obtained, the reversibility of the effects produced by a given medium was tested by comparing the densities of crystals before and after treatment with such a medium.

1—Methods

The protein crystals studied were obtained as described below. Edestin was prepared from hempseed which had been ground in a mill and thoroughly extracted with ether. The residual powder was dried in air and extracted with 10% sodium chloride, the temperature being slowly raised to 50°. The suspension was then filtered through a thick layer of paper pulp on a Buchner funnel and the clear filtrate was slowly diluted by the addition of an equal volume of water at 50°, with constant stirring, and allowed to stand. The solid deposited after about 16 hours at room temperature was wholly crystalline.

Horse serum albumin was prepared and recrystallized three times by the method of Adair and Robinson (1930). In some cases larger crystals were obtained by dialysis of an aqueous solution of serum albumin against a mixture consisting of 100 ml saturated ammonium sulphate, 60 ml M. ammonium acetate and 40 ml M. acetic acid. Egg albumin was prepared and recrystallized twice as described by Taylor, Adair, and Adair (1932).

Crystals of oxyhaemoglobin and CO haemoglobin of the sheep were prepared according to the method of Adair and Adair (1934), by the addition of ammonium sulphate. Crystals of horse haemoglobin were obtained by dialysis against distilled water, or by dialysis against dilute ammonium phosphate and by the addition of ammonium sulphate.

One native protein was examined in the amorphous state, namely, englobulin, which was prepared from horse serum by precipitation with one-third saturated ammonium sulphate. Denatured proteins were prepared by heat coagulation at the *iso*-electric point in the presence of salts.

In this work it was found that the most suitable media for the determination of the density of protein crystals were given by mixtures rather than solutions of single salts. Examples of the mixtures used are given in Table I. With the citrates and phosphates used, the proportions of acid and base were varied in order to study the effects of hydrogen-ion concentration on the crystal density.

It must be remembered that the proteins were usually crystallized by the use of salts other than citrates or phosphates. In order to eliminate such salts, the mixture of mother liquor and crystals was centrifuged, the mother liquor was poured off and replaced by citrate (or phosphate) solution of density greater than that of the crystals of protein. As a general rule, inorganic crystals containing ions from the mother liquor are formed. On centrifuging, the inorganic crystals sink and the protein

TABLE I—COMPOSITION OF THE SOLUTIONS USED FOR THE PREPARATION OF MEDIA

Two letters are used to designate media prepared from two solutions. A single letter denotes media prepared from a concentrated solution and distilled water. Density values in brackets. M. = gm mols/l solution.

AB A = Half-saturated ammonium sulphate.
B = Solution A saturated with sucrose.
CD C = Saturated ammonium sulphate (1·243).
D = Solution C saturated with sucrose (1·396).
EF E = Solution C adjusted to p_H 6·8 by addition of ammonia.
F = Solution E saturated with sucrose.
GH G = 100 ml saturated ammonium sulphate, 40 ml M. acetic acid, 60 ml M. ammonium acetate. p_H 4·8.
H = 50 ml G + 90 gm sucrose.
IJ I = 100 ml saturated ammonium sulphate, 20 ml M. acetic acid, 30 ml M. acetate. p_H 4·8.
J = 50 ml I + 90 gm sucrose.
K 100 gm $NaH_2PO_4 . 2H_2O$ + 100 ml H_2O. p_H 3·2 (1·334).
L 100 ml solution K + 10 ml 10 M. NaOH. p_H 5·0 (1·358).
N 170 gm potassium citrate $K_3C_6H_5O_7 . H_2O$ + 100 ml H_2O. p_H 8·3 (1·499).
O 150 gm potassium citrate, 7·62 gm citric acid, 105 ml H_2O. p_H 6·6 (1·460).
P 170 gm potassium citrate, 110 gm citric acid, 200 ml H_2O. p_H 4·3 (1·377).
QR Q = 0·005 M. $NH_4H_2PO_4$ + 0·005 M. $(NH_4)_2HPO_4$. p_H 7·0.
R = Solution Q + sucrose. (1·299).
S 50 ml solution L + 50 ml solution Q.

crystals float. The layer of protein crystals could then be removed and washed several times with the medium on the centrifuge.

The crystal paste was then mixed with about 25 cc of the medium and a series of centrifuge tubes was prepared with a constant volume of the suspension and varying amounts of water or of a solution lighter than the crystals. After the contents of the tubes had been mixed by repeated inversion, they were centrifuged at 2500 to 3000 r.p.m. for periods from 5 minutes to 45 minutes, according to the size of the protein crystals. After centrifugalization, the presence or absence of a sediment of crystals at the bottom of each tube was noted, the temperature of the fluid was measured and the density was determined at the same temperature.

The measurements of density were made with a pyknometer in the form of a pipette of capacity 3 ml fitted with a tap; the capillary tube above the bulb was graduated in cmm to facilitate measurements of the changes in volume and density with temperature. In order to prevent occlusion of the tip of the pyknometer, it was found advisable in experi-

ments with denatured proteins to free the liquid from suspended particles by forcing a plug of cotton wool to the bottom of the centrifuge tube by means of a glass rod, a procedure which is more rapid than filtration. The weights have been corrected for the air displaced and the densities are expressed in gm/ml, the density of water at 4° being taken as unity.

The following observations show that it is possible to work with a series of media differing by approximately 0·001 units in density. In preparing such mixtures, it may be advisable to weigh the samples of the medium of greater density, which is mixed with the protein crystals; and then to add the lighter fluid by means of a micro-burette. In a series of mixtures of saturated ammonium sulphate solution and of the same solution saturated with sucrose, the densities observed after centrifugalization were 1·2668, 1·2685, 1·2689, 1·2704, 1·2707, and 1·2740. In the first tube, with the lowest density, all of the crystals of sheep haemoglobin deposited; in the second and third tubes some protein crystals deposited and some remained suspended; in the last three tubes, no crystals deposited and a layer of crystals was observed at the surface. It may be inferred from these observations that the density is between 1·2689 and 1·2704.

In working with an ordinary centrifuge, 0·001 units in the density is near the limit of accuracy attainable, as the temperature rises 1–3° during centrifugalization. A number of measurements was made in which the crystals were allowed to settle by gravity at a temperature accurately controlled by a thermostat. If large crystals can be prepared, this method is more accurate, but the centrifuge is preferable for work with very small crystals.

The observations recorded in Table V show that it is possible to alter the densities of protein crystals by about 3% by changes in the composition of the medium in which the crystals are suspended. The existence of such a change shows that it is necessary to specify the medium, but as the change is small, it seems permissible to abbreviate the experimental work and the tabulated records by the following procedure. In all experiments, a pair of solutions is used, one of density greater and one of density less than that of the protein crystals, in some cases distilled water being the lighter liquid. At a given temperature, the heavier and the lighter solutions must be mixed in definite proportions in order to give a specified density, and therefore the series of measurements recorded in Tables V, VI, and VII, in conjunction with the data for the composition of the stock solutions given in Table I, serves not only to show the density of the protein but also to define the composition of the medium, which could be estimated in terms of concentrations, if required, by preparing

a series of mixtures of the two solutions and plotting the concentrations of ions (and other properties such as vapour pressure) against the densities of the mixtures.

A similar procedure could be applied to estimate p_H values, at different densities, using a hydrogen electrode and a saturated solution of potassium chloride to reduce the liquid junction potentials. In concentrated solutions containing ions of different mobilities, the uncertainties due to the liquid junction potentials may exceed 0·1 p_H units. Detailed investigations of the p_H of all of the salt solutions employed in the density determinations have not been made. Some p_H values for undiluted solutions, measured with the hydrogen electrode at 20°, or at 30°, supplemented by uncorrected colorimetric measurements, are recorded in the tables.

It should be noted that the p_H values for diluted mixtures in which the protein crystals are suspended are not necessarily equal to the figures recorded for undiluted solutions. The following examples show the very considerable effects of dilution. A 4·3 M. solution of NaH_2PO_4 of density 1·334 and p_H 3·2 was diluted to M./15. After dilution, the density was 1·0035 and the p_H 4·5. In a second solution, NaH_2PO_4 3·67 M. + Na_2HPO_4 1·25 M. the density was 1·425 and the p_H 4·82. In a mixture containing 6 ml of this solution and 4 ml water, the density was 1·27 and the p_H 5·16. After dilution to M/15, the density was 1·004 and the p_H was 6·38.

2—Validity of the Experimental Method

There is a number of possible sources of error in the experimental method, including irreversible changes which might be caused by the concentrated solutions in which the crystals are suspended, the occlusion of mother liquor, and the existence of films of liquid at the surface of the crystals.

It was found that, in general, the protein crystals suspended in the media listed in Table I could be redissolved in dilute salt solutions, an observation which shows that the media do not cause irreversible changes which are characterized by the formation of derivatives insoluble in dilute salt solutions. With edestin crystals suspended in a citrate medium at p_H 4.3, a small part of the protein had been converted into an insoluble form. Larger amounts of insoluble products were obtained in experiments with ammonium nitrate and rubidium chloride, which have not been recorded.

Additional experiments were made on haemoglobin in phosphate mixtures, after the effects of dilution on the p_H values of such mixtures

had been determined by means of the hydrogen electrode, as stated in § 1. The proportions of NaH_2PO_4 and Na_2HPO_4 had been adjusted to give p_H values from 6·2 to 6·4 in dilute solutions of density 1·004, but if the densities be increased to 1·22, the p_H values may fall to 5·2 or 5·0. Haemoglobin solutions may undergo partial denaturation in dilute buffer solutions of such p_H values.

It was found that when a solution of sheep oxyhaemoglobin was mixed with an excess of the concentrated phosphate mixture of p_H 5·2, crystals were formed on standing for a few hours at room temperature. The crystals changed gradually in colour from red to brown. After a few days, the solution was diluted with water and the crystals dissolved without leaving any residue. Spectroscopic examination showed that methaemoglobin was present. Reduced haemoglobin was formed on the addition of sodium hydrosulphite and oxyhaemoglobin after shaking the reduced haemoglobin with air. These observations show that the protein is not denatured under the given conditions. In the determinations of crystal density, CO haemoglobin was used rather than oxyhaemoglobin, which is more readily converted into methaemoglobin. Preliminary experiments showed no difference between the densities of pure CO haemoglobin crystals and of crystals which contain some methaemoglobin.

Even if there are no obvious signs of denaturation, it is desirable to make sure that the crystals are not being gradually injured by the medium and that a state of equilibrium has been reached, by measuring the densities of crystals which have been exposed to the medium for different periods of time. In the centrifugal method, the crystals need not remain in the medium for more than 20 minutes, without the centrifuge several hours are required. The results summarized in Tables II and III represent observations carried out through periods varying from a few hours to two days, but in all cases the densities are the same, within the limits of error. The series of experiments includes determinations on protein crystals prepared under different conditions, giving microscopic and visible crystals, and, for sheep haemoglobin, crystals freshly prepared and material stored at $-10°$ for months. The experiments indicate that the density is a constant, independent of the time the protein is exposed to the medium within the limits defined by the "differences" given in the last column of Table II.

The densities and differences are calculated by the following procedure. If D_s be the density of the more concentrated solution in the series where sedimentation took place, and D_f be the density of the least dense solution where no sediment of crystals was observed, the true density should be

intermediate between D_s and D_f. Instead of tabulating the absolute values of D_s and D_f, it is more convenient to record the mean D, equal to $\frac{1}{2}(D_s + D_f)$ and the "difference" $D^{\pm}$, equal to $D - D_s$ or $D_f - D$. It is obvious that in a series of experiments where the densities are not closely spaced, the difference $D^{\pm}$ is subject to a much wider range of variation than the experimental errors in measurements of the densities of the solutions.

TABLE II—DENSITY OF CRYSTALS OF HAEMOGLOBIN

Medium AB half-saturated ammonium sulphate + sucrose.
Medium CD saturated ammonium sulphate + sucrose.
Medium EF saturated ammonium sulphate + sucrose, p_H 6·8.

Species	Medium	Temperature °C	Density	Difference
Horse	AB	19·0	1·2699	0·0001
Horse	AB	19·9	1·2700	0·0040
Horse	AB	20·0	1·2700	0·0020
Sheep	CD	22·0	1·2696	0·0010
Sheep	EF	20·8	1·2669	0·0027
Sheep	EF	19·8	1·2642	0·0010
Horse	EF	17·9	1·2656	0·0016

The Tables II and III give a complete record of the data, including series where the densities were not closely spaced, and it may be seen that in all preparations the range of variation in the mean value D can be accounted for by the range in the "differences".

TABLE III—DENSITY OF CRYSTALS OF HORSE SERUM ALBUMIN

Medium GH half-saturated ammonium sulphate + sucrose, p_H 4·8.
Medium IJ two-thirds saturated ammonium sulphate + sucrose, p_H 4·8

Medium	Temperature °C	Density	Difference
GH	16·5	1·2760	0·0060
GH	18·2	1·2739	0·0005
GH	18·0	1·2719	0·0025
GH	18·0	1·2725	0·0025
IJ	18·0	1·2751	0·0029
IJ	18·0	1·2784	0·0018
GH	0·0	1·2834	0·0016

If, however, the protein crystals be suspended in media of different composition, their density may be changed by an amount markedly greater than the "differences". In Table IV, experiment 1 shows that

crystals of haemoglobin suspended in sodium phosphate have a density of 1·2269. The density is increased to 1·2642 when the crystals are suspended in a mixture of sucrose and ammonium sulphate and reverts to 1·2268 when the same crystals are washed and re-suspended in the sodium phosphate solution.

The experimental evidence for the reproducibility and the reversibility of the results shown in Tables II, III, and IV would seem to show that if suitable media be chosen, the method gives valid results, if it be understood that the observed density is a function of the composition of the liquid in which the crystals are suspended.

TABLE IV—REVERSIBILITY OF THE CHANGE OF CRYSTAL DENSITY OF SHEEP HAEMOGLOBIN CAUSED BY THE MEDIUM

Experiment No.	Medium	Temperature °C	Density	Difference	Notes
1	$K + H_2O$	19·7	1·2269	0·0020	Crystals in absence of sucrose
2	EF	20·7	1·2642	0·0010	Sucrose and $(NH_4)_2SO_4$
3	$K + H_2O$	20·2	1·2268	0·0025	Crystals used in expt. 2

Reilly and Rae (1933) state that when organized substances such as cotton fibre are submitted to what are called density determinations, the results are purely empirical, the quantity of the medium adsorbed depending on its viscosity and on many other external conditions. The densities of different specimens of charcoal (measured in water) range from 1·17 to 1·63.

Although the densities of protein crystals are affected by the composition of the medium, the experiments recorded above show a relatively high degree of constancy and reproducibility. The difference between the protein crystals and ordinary porous substances may be accounted for on the hypothesis that the protein crystals are highly permeable, and rapidly attain a state of thermodynamic equilibrium with the aqueous solution in which they are suspended. It is probable that all of the constituents of the media used in this work can diffuse into protein crystals, which should be regarded as a solid phase containing protein, rather than a pure substance. The composition of this phase and its density should be a function of the composition of the liquid (practically free from protein) in which the crystals are suspended, and it follows that the crystals should be thoroughly washed with this liquid, otherwise the results will be in some degree affected by the electrolytes present in the mother liquor.

3—The Densities of Crystals of Edestin, Serum Albumin, Egg Albumin, Haemoglobin, and of Globulin

Observation of the densities of protein crystals are summarized in Tables V, VI, and VII. In order to facilitate the comparison of the results of experiments made at slightly different temperatures, all of the densities have been reduced to 20°, assuming that a rise in temperature of 1·0° reduces the density by 0·0008 units. This value is based on

Table V—Densities of Crystals of Edestin, Horse Serum Albumin, Egg Albumin, and of Horse Serum Euglobulin

Media described in Table I. D = density reduced to 20°. T = temperature of measurement. w = provisional estimate of hydration in gm water per gm dry protein (ref. formulae 2 and 8).

Protein	D	Difference	w	Temperature °C	Medium
Edestin	1·317	0·002	0·063	21·1	CD. Saturated ammonium sulphate and sucrose
,,	1·288	0·001	0·143	22·0	L. Phosphate p_H 5·0
,,	1·288	0·003	0·143	24·0	N. Citrate p_H 8·3
,,	1·290	0·004	0·137	20·9	O. Citrate p_H 6·6
,,	1·308	0·003	0·086	24·4	P. Citrate p_H 4·3.
Serum albumin	1·276	0·003	0·192	18·0	GH. Half-saturated $(NH_4)_2SO_4$ and sucrose p_H 4·8
,,	1·279	0·002	0·182	18·0	IJ. Two-thirds saturated, $(NH_4)_2SO_4$ and sucrose p_H 4·8
,,	1·246	0·001	0·304	20·8	O. Citrate p_H 6·6
,,	1·237	0·006	0·344	20·3	L. Phosphate p_H 5·0
,,	1·278	0·001	0·185	21·5	K. Phosphate p_H 3·2
Egg albumin	1·239	0·001	0·324	19·0	L. Phosphate p_H 5·0
,,	1·268	0·002	0·208	20·0	K. Phosphate p_H 3·2
Globulin	1·236	0·006	0·331	20·0	L. Phosphate p_H 5·0

observations of the density of haemoglobin in solution, and may require revision when direct determinations of the effect of temperature on crystal density have been made. A preliminary measurement on horse serum albumin gave a temperature coefficient somewhat below 0·0008.

A number of conclusions can be drawn from the measurements of the densities given in Tables V, VI, and VII:—

(1) In all cases the density of the crystals is definitely below the apparent density of the anhydrous protein in an aqueous solution, determined by Svedberg as approximately 1·34.

TABLE VI—DENSITIES OF CRYSTALS OF HORSE AND OF SHEEP HAEMOGLOBIN

Species	D	Difference	w	Temperature °C	Medium
Horse	1·269	0·000	0·166	19·0	AB. Half-saturated $(NH_4)_2SO$ + sucrose
,,	1·264	0·002	0·183	17·9	EF. $(NH_4)_2SO_4$ + sucrose p_H 6·8
Sheep	1·264	0·001	0·183	20·8	EF. $(NH_4)_2SO_4$ + sucrose p_H 6·8
Horse	1·267	0·001	0·172	17·9	QR. 0·01 M phosphate + sucrose
,,	1·252	0·002	0·227	17·9	S. NaH_2PO_4 + sucrose
,,	1·225	0·004	0·344	20·0	L. Phosphate p_H 5·0
Sheep	1·226	0·001	0·338	19·7	L. Phosphate p_H 5·0
,,	1·231	0·002	0·315	20·4	O. Citrate p_H 6·6
,,	1·242	0·001	0·267	22·0	Saturated $(NH_4)_2SO_4$ + H_2O
Horse	1·238	0·003	0·284	24·5	Saturated $MgSO_4$ + H_2O

TABLE VII—COMPARISON OF DENSITIES OF PROTEIN CRYSTALS AND OF PROTEINS AFTER HEAT COAGULATION

Protein		D	Difference	w	Temperature °C	Medium
Sheep haemoglobin	Coagulated	1·260	0·001	0·197	21·6	QR. Sucrose
Horse haemoglobin	Crystals	1·267	0·001	0·172	17·9	,,
,,	Coagulated	1·263	0·003	0·187	19·4	,,
,,	Crystals	1·225	0·004	0·344	20·0	L. Phosphate p_H 5
,,	Coagulated	1·229	0·002	0·324	20·1	,,
Egg albumin	Crystals	1·239	0·001	0·324	19·0	,,
,,	Coagulated	1·258	0·001	0·245	22·0	,,
,,	Crystals	1·268	0·002	0·208	20·0	K. Phosphate p_H 3
,,	Coagulated	1·280	0·002	0·168	23·3	,,

(2) The densities of different proteins suspended in solutions containing the same salts show a marked degree of specificity, whereas the apparent densities of the anhydrous proteins show comparatively small variations, as shown in Tables VIII and IX.

It will be observed that edestin, a plant globulin, has a much higher density than any of the animal proteins investigated, which include precipitated englobulin prepared from horse serum.

(3) In comparing the densities of the same protein crystals suspended in different media, it was noted that the figures obtained with mixtures of ammonium sulphate and sucrose were above those obtained with either phosphates or citrates. Further experiments with haemoglobin crystals indicated that in pure ammonium sulphate the density is low, and in sucrose solutions containing only a trace of electrolyte, the density is high. Intermediate values were obtained by mixing sodium, phosphate, and sucrose. In view of this evidence, recorded in Table VI, it would seem that sucrose has a specific effect in raising the density of the crystals.

(4) In mixtures, containing citrates and phosphates, a fall in p_H value is correlated with an increase in the density.

(5) The density of proteins denatured by heat coagulation is not very much less than that of the crystals, although with egg albumin there is a change greater than the experimental error.

4—The Apparent Densities and Specific Volumes of Anhydrous Proteins in Solution

An extensive series of measurements of the specific volumes of proteins has been included in the publications of Svedberg and his colleagues, including the work of Svedberg and Stamm (1929) on edestin, Svedberg and Sjögren (1928) on horse serum albumin and globulin, and Nichols (1930) on egg albumin.

For the comparison of the densities of the crystals and the apparent densities of proteins in solution, it is desirable that the proteins should be prepared and the measurements of densities standardized by the same procedure, and therefore a number of measurements of the apparent densities of the type described by Svedberg has been made in this work, and recorded in Table VIII. In these measurements a known mass and volume of a pure aqueous solution of the protein of known density was dried to constant weight, in some experiments at 110° and in others in a high vacuum over phosphorus pentoxide at 0°. The volume of the pro-

tein could then be calculated by subtracting the volume of the water, estimated from the mass and the density of pure water, from the volume of the solution. It was found that the apparent specific volume of a given protein calculated from these measurements was a constant, independent of the protein concentration.

According to the definition given by Lewis and Randall (1932), the "partial specific volume" of the dry protein, symbolized v_p, is the increase in the volume of a large mass of solution, caused by the addition of 1 gm of the dry protein, as stated in formula (1)

$$v_p = (\partial V / \partial m_p)\ P, T, m \qquad (1)$$

V = volume of solution; m_p = mass of anhydrous protein. The letters P, T, and m denote that the pressure, temperature, and the masses of all substances, except the protein, are constant.

It follows from the definition that if the apparent specific volume be constant, the partial specific volume is a constant and equal to the apparent specific volume. It may be noted that whereas Svedberg's values for the specific volumes of serum albumin and haemoglobin are the same, our values show a difference of 0·01 cc (Table VIII). The measurements of the densities of the crystals show a difference of the same order.

TABLE VIII—PARTIAL SPECIFIC VOLUMES OF PROTEINS IN AQUEOUS SOLUTIONS

	Temperature °C	Volume	Density
Sheep haemoglobin	0	0·7431	1·3457
,,	16	0·7506	1·3322
,,	20	0·7524	1·3290*
Horse serum albumin	20	0·7418	1·3481*
Egg albumin	20	0·7457	1·3410
Water	20	1·0018	0·9982*

* Ref. formula 8. The density given in this table, and symbolized D_p in formula 8 is defined as the reciprocal of the partial specific volume of the anhydrous protein in solution. D_p may be different from the density of the proteins in the dry state.

In reviewing the data in Tables VIII and IX, it will be seen that edestin has the highest apparent density, and haemoglobin the lowest; but the range of variation is small, from 1·346 to 1·329, a range of 1·35%. The densities of crystals of edestin and haemoglobin in citrate buffers are in the same order, but the range is from 1·290 to 1·231 or 4·5%.

In comparing the effects of electrolytes on the apparent density of a given protein, the maximum change observed is 0·009 units, whereas the densities of crystals show a range of 0·043 units.

TABLE IX—PARTIAL SPECIFIC VOLUMES OF PROTEINS DISSOLVED IN DIFFERENT MEDIA, TEMPERATURE ABOUT 20° C

Protein	Volume	Density	Medium
Edestin†	0·744	1·346*	NaCl 1·24 M. p_H 5·5
,,	0·745	1·342	NaCl 0·62 M. p_H 11·3
Egg albumin‡	0·749	1·335	Water
,,	0·744	1·344	Phosphate 0·017 M. p_H 7·3
,,	0·749	1·335	Acetate 0·02 M. p_H 4·7
,,	0·744	1·344*	NaCl 0·17 M.
Serum globulin§	0·745	1·342	
Serum albumin§	0·748	1·337	
Haemoglobin	0·749	1·336	p_H 5·4
,,	0·753	1·328	p_H 9·0

* Ref. formula 8.
† Svedberg and Stamm (1929).
‡ Nichols (1930).
§ Svedberg and Sjögren (1928).
‖ Svedberg and Nichols (1927).

5—THE COMPOSITION OF PROTEIN CRYSTALS

Sørensen (1917) showed that protein crystals cannot be separated from the mother liquor, without risk of alteration. Complete analyses giving the proportions of all of the substances, including water and salts, that may be present in the crystal cannot therefore be made.

If, however, there is a higher proportion of either water or salt in the crystal than in the mother liquor, it is possible to obtain estimates of the substance which is present in excess by means of analyses of the filtered mother liquor and of precipitates containing crystals and adherent mother liquor as described by Sørensen (1917). Determinations of this type are of importance for the interpretation of the measurements of the densities of the crystals, since an excess of water in the crystal may give a density below that of the dry protein.

The results obtained by previous workers, summarized in Table XI, all showed that the protein crystals contain a higher ratio of water to salts than the mother liquor. Adair and Adair (1934) found that if crystals of horse CO haemoglobin be equilibrated with a dilute solution of electrolytes (0·01 molar ammonium phosphates) instead of the highly concentrated solutions of salts, including half-saturated ammonium sulphate used by Sørensen and his colleagues, the distribution of water and salts is altered, so that the proportion of ammonium phosphate was higher in the crystals than in the mother liquor or in the ultra-filtrate.

In the case of one experiment, recorded in Table X, the analytical results summarized by Adair and Adair (1934) have been stated in greater detail, and expressed in gm or gm mol/1000 gm of "total water". It will be seen that there is an excess of salts in the mixture containing crystals, but that the quantity does not exceed 1·29 gm 372 gm of haemoglobin.

It is not possible to estimate the amount of water in protein crystals equilibrated with dilute salt solutions by the method of Sørensen (1917) since the apparent hydration is negative for crystals which contain an excess of salts; but even under these conditions there is evidence that the protein crystals contained water of hydration. In Tables VI and VII experiments are reported in which the density of haemoglobin crystals

TABLE X—CRYSTALS OF HORSE CO HAEMOGLOBIN IN EQUILIBRIUM WITH 0·005 M. $NH_4H_2PO_4$ + 0·005 M. $(NH_4)_2HPO_4$. p_H 7·09 AT 0° C

Weights of components in grammes. 4% of total protein dissolved, 96% in crystals

Phase	H_2O	NH_4	PO_4	Protein	Density
(1) Crystal + mother liquor ..	1000	0·32	2·19	372·0	1·0755
(2) Medium (ultra-filtrate)	1000	0·27	0·95	0·0	1·0007
Excess ions in phase (1)	—	0·05	1·24	—	—
Excess in mols/mol haemoglobin (67,000 gm)	—	0·50	2·30	—	—

was measured in the presence of 0·01 molar ammonium phosphate and sucrose. Even though sucrose tends to increase the density of protein crystals and to reduce the apparent hydration calculated from such measurements as described below, the density of the crystals in this medium was less than that of the anhydrous protein.

In the range of concentrations investigated by previous workers, where an excess of water rather than of salt was present in the crystal the hydration of the protein can be estimated as described by Sørensen (1917). A simplified form of this method is outlined below. The crystal, composed of protein, water, and salt, can be regarded as a mixture of two components, A and B, where B is a mixture of water and salt in the proportions which obtain in the dispersion medium, and A is a mixture of protein and water.

In a chemical analysis giving the proportions of protein, water, and salt in mixture of crystals and a dispersion medium free from protein, it is not possible to differentiate between component B and the adherent dis-

persion medium, but the sum of masses of water existing in the forms of dispersion medium and component B can be computed by multiplying the weight of salt by the ratio of water to salts determined by an analysis of the dispersion medium obtained by filtration (or by ultra-filtration). This sum subtracted from the total water gives the mass of water existing in the form of component A.

Sørensen (1917) has shown that under certain conditions, it is probable that the mass of component B is small, and that the water content of component " A " cannot be much less than the total water present in the crystal, but for the purpose of the present work, the consideration of the total water content of the crystal may be postponed, because values of the hydration defined as the water content of component A, which can be obtained by direct analyses, can be used for the interpretation of the measurements of densities.

Sørensen (1917) has given formulae by means of which the hydration can be calculated from analyses expressed in ammonia and protein nitrogen, without converting the results into grams of salt, water, and protein.

$$w = (r - r_a)/r_a \tag{2}$$

w = apparent hydration in gm water per gm anhydrous protein (component A = 1 gm protein + w gm H_2O). r_a = gm anhydrous protein per gm protein nitrogen. r = g protein hydrate per gm protein nitrogen. The number r is computed from measurements of ammonia and protein nitrogen in filtrate and precipitate.

Sørensen used this formula to calculate the hydration of the protein crystal in the case of egg albumin equilibrated with ammonium sulphate, but the majority of his determinations are recorded in terms of r. In Table XI the values of r have been collected and used for the calculation of w. For egg albumin we have used Sørensen's value of r_a. For the blood proteins, Sørensen did not determine r_a, and we have made a number of determinations in order to complete the calculation of w. The value of r_a for total globulin has been recorded, as that for the pseudo-globulin fraction studied by Sørensen is not known.

For haemoglobin, the value of $r_a = 5 \cdot 78$ formerly accepted yields $w = 0 \cdot 353$. (Sørensen and Sørensen, 1933). The value $w = 0 \cdot 306$ given in the table is based on the more recent figure for r_a of Vickery and Leavenworth (1928), which is in accordance with our analyses.

6—Theoretical Relations Between the Composition and the Density of Protein Crystals

The derivation of formulae correlating the density and the composition of the protein crystal is of importance for testing hypotheses which attribute the relatively low density of the protein crystal to hydration.

Since the evidence available indicates that, within certain limits, the proportions of ammonia, phosphoric acid, and water in the crystals can be varied continuously by altering the composition of the dispersion

Table XI—Hydration of Crystalline, Precipitated, and Coagulated Proteins by Sørensen's Method of Proportionality

Protein	Medium	p_H	r	r_a	w
Egg albumin crystals	Ammonium phosphate	4·8	7·89* ..	6·4†**	0·233
,, ...	,,	5·5	8·12* ..	6·4‡**	0·269
,, ...	Ammonium sulphate	—	7·86†	6·4‡**	0·228‡
Egg albumin coagulated	,,	—	7·65‡	6·4‡**	0·195
,,	Sucrose	—	7·49‡	6·4‡**	0·171
Serum albumin crystals	Ammonium sulphate	—	8·35§	6·41††	0·303
Pseudo-globulin precipitated	,,	—	8·88§	6·61†‡	0·343
Globulin (ox)	,,	—	8·74‖		
Albumin (ox)	,,	—	8·31‖		
Horse haemoglobin crystals	,,	—	7·82¶	5·99‡‡	0·306

* Sørensen and Palitzsch (1923).
† Sørensen (1917).
‡ Sørensen and Sørensen (1924).
§ Sørensen (1925).
‖ Bonot (1934).
¶ Sørensen and Sørensen (1933).
** Taylor, Adair, and Adair (1932).
†† Adair and Robinson (1930).
‡‡ Vickery and Leavenworth (1928).

medium (Adair and Adair, 1934), it may be concluded that the formulae for the specific volumes of solutions, given by Lewis and Randall (1923) must be applicable to the protein crystals.

According to these authors, the volume of a mixture of n substances, designated S_1, S_2, S_3, ..., S_n, should be equal to the sum of the masses of each of the substances multiplied by their partial specific volumes, defined

by formula (1). In the case of a protein crystal, the specific volume may be represented by formula (3).

$$v = x_p v_p + x_1 v_1 + x_2 v_2 + \dots x_n v_n \tag{3}$$

v = specific volume (ml per gm protein crystal), x_p = weight fraction of anhydrous protein (gm/gm crystal). x_1, x_2, x_n = weight fractions of substances S_1, S_2, S_n, v_p = partial specific volume of anhydrous protein, v_1, v_2, v_n = partial specific volumes of S_1, S_2, S_n.

A formula more directly applicable to the available experimental data can be obtained, at the cost of a loss in generality and a slight sacrifice in accuracy, by the following procedure.

Consider a system where the protein crystals are suspended in a liquid of the same density, composed of two substances, S_1 = water, and S_2 = a substance with a relatively high density. The specific volume (v'') of this dispersion medium must be determined by formula (4)

$$v'' = x_1'' v_1'' + x_2'' v_2'', \tag{4}$$

x_1'' and x_2'' = weight fractions, v_1'' and v_2'' = the partial specific volumes of S_1 and S_2 in the dispersion medium.

The specific volume of the protein crystal in equilibrium with this medium is given by formula (5)

$$v = x_p v_p + x_1 v_1 + x_2 v_2. \tag{5}$$

Such a crystal can be regarded as a mixture of two components, A and B, where the component B is a mixture of S_1 and S_2 in the ratio x_1''/x_2'' which obtains in the dispersion medium, and the component A is composed of anhydrous protein and water, as stated in the previous section.

It is reasonable to assume that the specific volume of the component B, v_B, is approximately equal to the specific volume of the dispersion medium. In a measurement of crystal density, the specific volume of the crystal as a whole is equal to that of the medium and therefore it is legitimate to make the approximate assumption that

$$v_A = v_B = v'' = x_p v_p + x_1 v_1 + x_2 v_2 \tag{6}$$

v_A the specific volume of component A, a mixture containing w gm water per gm dry protein.

If x be the weight fraction of water and $1 - x$ the weight fraction of anhydrous protein, the specific volume of the component A should be determined by formula (7)

$$v_A = (1 - x)\, v_p + x v_1. \tag{7}$$

It is evident that if v_p and v_1 be known, x can be calculated by means of this formula, since v_A is approximately equal to the specific volume of the crystal as a whole. If no direct determinations of v_p and v_1 be available, provisional estimates can be made, by assuming that v_p be the same as v_p' the partial specific volume of the protein in an aqueous solution, and that v_1 be equal to the specific volume of pure water.

It is possible to make a simple algebraic transformation of formula (7) which saves a considerable amount of time in arithmetical calculations. Let w = gm water of hydration per gm dry protein defined by the formula $w = x/(1 - x)$. By formula (7), the ratio

$$x/(1 - x) = \left(1 - \frac{v_p}{v}\right) \Big/ \left(\frac{v_1}{v} - 1\right)$$

The specific volumes can then be replaced by the densities, as stated in formula (8).

$$w = \left(\frac{D_p - D}{D - D_1}\right) \frac{D_1}{D_p}. \tag{8}$$

D = density of crystal, D_p = density of anhydrous protein = $1/v_p$, D_1 = density of water. A series of values of w calculated by this formula is given in Tables V, VI, and VII. The values of D_p used for these calculations are marked with an asterisk in Tables VIII and IX. These calculations must be regarded as provisional, since the values for D_p and D_1 now available were obtained on solutions of proteins in media of low density. The range of variation in the values of D_p caused by alterations in the medium is not great, as shown in Table IX, but it may cause errors exceeding 0·02 in the estimation of w. Many of the liquids used are systems of three or more components, where the specific volume must be determined by the more general formula (3).

One example may be given to illustrate the conditions in liquids containing three or more components. In the case of the experiment (Table VI) where crystals of horse haemoglobin have been suspended in a mixture of sucrose and ammonium phosphate, an estimate of the effects of the phosphate can be obtained by using the data given in Table X. The crystal contains more phosphate than the dispersion medium. The excess is equivalent to 0·0035 gm per gm dry protein. Assuming that the specific volume of the salt is 0·55 and that of the protein is 0·75, the change in the specific volume due to the excess of salt is below 0·001 units, an amount which is less than the experimental errors.

Discussion

The observation that the densities of the protein crystals investigated in this work are less than the apparent densities of the dry solids has been discussed in the previous sections in accordance with the hypothesis that the protein crystals contain water of hydration. An alternative hypothesis might be put forward, namely, that there is a contraction of volume when the protein crystals are dissolved. Two instances might be cited in favour of this second hypothesis:—

(1) There is a well-marked contraction of about 0·05 cc per gm when dry gelatine absorbs water.

(2) Chick and Martin (1913) measured the density of dried egg albumin, serum albumin, and pseudo-globulin immersed in benzene and obtained the values 1·269, 1·275, and 1·29 ± 0·02 respectively. The solution contraction volumes estimated from these figures are of the order of 0·04 ml per gm (Cohn, 1935).

These observations are suggestive but inconclusive, because the contraction when dry protein is dissolved is not necessarily equal to the contraction when protein crystals are dissolved.

Since we have not been able to find any published evidence bearing on this problem, a series of experiments was made to determine the partial specific volume of the protein, existing in the form of edestin crystals dialysed against distilled water, and horse haemoglobin crystals prepared by dialysis against distilled water.

The contents of pyknometers containing a known weight and volume of the suspensions were washed into platinum basins, dried, and weighed, and the specific volumes were calculated as for solutions. In some experiments, the pyknometers were washed out with dilute alkali and the protein content was thus determined by the method of Kjeldahl. The results of two experiments are recorded in Table XII.

The contraction in volume when the crystal dissolves can be calculated by subtracting the apparent specific volume of the protein in solution from the apparent specific volume of the protein in the form of a solid suspension. The experiments show a change of the order of ± 0·003 ml per gm dry protein, which is of the same order as the experimental errors.

In considering the question whether the relatively low density of protein crystals is due to a contraction in volume on solution, the experiments recorded in Table XII have a more direct bearing on the problem than the experiments on gelatine or on proteins in benzene referred to above, and therefore the hypothesis may be rejected.

The alternative hypothesis that the low densities of protein crystals are due to water of hydration has been tested by deducing a formula (No. 8) for the calculation of the water of hydration from measurements of the density of the crystal, and then comparing the calculated values with the results obtained by Sørensen's method. In comparing the two sets of data, recorded in Tables V, VI, VII, and XI, exact agreement cannot be anticipated for two reasons. The apparent hydration w varies to a certain extent with the p_H value, the vapour pressure, and the concentrations of inorganic ions. All of the available data on the composition of the crystals obtained by Sørensen's method refer to systems where the concentration of salts is lower than those used in the experiments on crystal densities. In the second place, the measurements of the specific

TABLE XII—THE PARTIAL SPECIFIC VOLUME OF DRY PROTEIN IN SUSPENSION AND IN SOLUTION

Protein	Horse haemoglobin	Edestin
Temperature ° C	1·0	21·9
Apparent density of dry protein in the form of solid suspension in distilled water, wholly crystalline in the case of haemoglobin	1·344	1·349
Partial specific volume of dry protein in the form of suspension	0·744	0·741
Partial specific volume of dry protein in solution (ref. Tables VIII and IX)	0·742	0·745
Contraction in ml/gm crystalline protein dissolved	0·002	−0·003

volumes of dry protein and of water have been made at low concentrations of electrolytes, and all of the calculations of hydration from measurements of the densities by formula (8) must be regarded as provisional values.

In reviewing the data on different proteins and different media, it will be seen that the experiments can be divided into two main groups. In the first group, which includes experiments with concentrated salt solutions such as phosphates and citrates, free from sucrose, the values of w computed from densities rarely differ by much more than 0·03 units from the estimates based on chemical analyses at similar p_H values. Such small differences as occur are in the direction that might be anticipated, allowing for the differences in the p_H values and vapour pressures. The results indicate that under certain conditions the hydration theory, even in the simplified form that has been outlined, is concordant with the results obtained by the method of Sørensen.

In the second group, where solutions of ammonium sulphate similar to those used by Sørensen were employed, together with sucrose to raise the density to that of the protein crystals, the values of w calculated from densities may be as much as 0·1 gm below those calculated from chemical analyses. For horse serum albumin, for example, the apparent hydration is 0·303 gm calculated from Sørensen's analyses, and 0·19 gm from the measurements of densities by formula (8). These observations do not necessarily invalidate the formula, because the experimental conditions are not identical. A rigorous test of formula (8) would necessitate chemical analyses with sucrose as well as ammonium sulphate as the reference substance.

The apparent discrepancy might be accounted for by two mechanisms, namely, the removal of water from the crystals when sucrose is added to the medium in which they are suspended, or the absorption of sucrose by the crystals. The possibility that serum albumin and egg albumin may have a slight affinity for glucose is shown by the measurements published by Weber and Nachmannsohn (1929) and by Versmold (1931). In the case of gelatine, however, the replacement of electrolytes by sucrose as a reference substance tends to increase the apparent hydration (Moran, 1932).

The hypothesis that the difference between the densities of anhydrous protein and of protein crystals is due to hydration is not inconsistent with the second group of experiments and is strongly supported by the experiments on media free from sucrose. It seems probable that the measurement of crystal density may afford a valuable addition to the methods now available for the estimation of the hydration of the proteins.

In the simplest form of the method, used for the calculations recorded in Tables V, VI, and VII, three possible sources of error must be recognized:—

(1) The specific volumes of water and of anhydrous protein in the crystal phase may differ from the provisional values assumed, which are based on data for solutions.

(2) In systems where the medium contains three or more components in high concentrations, inequalities in the ratio salt/water in the crystal phase for different components may cause errors when formula (8) is used rather than formula (3).

(3) The measurements of crystal density in some cases are subject to errors of 0·003 units, which corresponds to an error in the value of w of 0·01 gm per gm protein.

In theory, the calculations of hydration from densities are not quite so accurate as calculations based on chemical analyses of filtrates and precipitates, but it must be remembered that quite small errors in the chemical analyses are greatly magnified in the calculation of w.

A brief reference may be made to two theoretical problems, namely, the mechanism by which water of hydration may be held, and the presence of water associated with protein molecules in solutions.

Jordan Lloyd and Phillips (1933) have described a number of methods by which water molecules might be coordinated to atoms on the side chains of a peptide. Some of their conclusions have been criticized by Pauli (1934).

Sidgwick (1927) has pointed out that care is required in the interpretation of measurements of the water of crystallization of salts. In general, the results are consistent with the theory that water and salt are chemically combined by a coordinate co-valence, but there is a number of cases of "excessive hydration" where uncombined water molecules may be present in the crystal. He refers to the crystalline hydrates of the inert gases.

In order to obtain evidence on this point, measurements of density and hydration have been made in this work on proteins denatured by heat coagulation, which converts the orderly crystalline arrangement into amorphous flocks. The data recorded in Table XIII show that heat-denatured haemoglobin retains 97% and heat denatured egg albumin 81% of the water present in the crystal. It seems that the greater part of the water is held by forces that are independent of an orderly crystalline arrangement of the molecules. The relatively small amount of water lost on heat coagulation may be attributable to a chemical change, since the denatured protein is an insoluble substance.

Evidence for a fall in hydration on heat coagulation has been obtained by two independent methods, namely, the chemical method of Sørensen and by the equilibration of finely divided protein with aqueous vapour from saturated salt solutions (Adair and Robinson, 1931). The data summarized in Table XIII show that the difference in hydration caused by denaturation determined by both of these methods agrees with the difference calculated from the measurements of crystal density. The absolute values differ as the p_H values and salt concentration in the systems were different.

It may be noted that if water be combined with the protein by chemical forces, including coordination, the measurements of the hydration of the crystals could be used to draw conclusions concerning the hydration of the protein in solution, but until the chemical theory has been worked out

in greater detail, it is preferable to discuss this problem from the experimental standpoint.

Although the quantities of water associated with one mol of the protein in the crystalline form and in solutions are not necessarily equal, there is a number of observations which support the hypothesis that water is associated or combined with protein molecules in solution as well as in the solid phase.

1—It has been shown that a number of inorganic ions, including phosphate and ammonium, diffuse freely into the protein crystal, and the environment of a protein molecule in the crystal thus corresponds closely to the environment when in solution. It is therefore reasonable to suppose that the degrees of hydration in the crystalline form and in solution are at least approximately equal.

TABLE XIII—EFFECT OF HEAT COAGULATION ON HYDRATION

Protein	Apparent hydration calculated from density	Hydration of solid protein water vapour over ammonium sulphate 81% humidity	Apparent hydration by the method of Sørensen
Haemoglobin (native)	0·34	0·21	0·306
,, (heat coagulated)	0·33	0·20	—
Egg albumin (native)	0·208	0·21*	0·228
,, (heat coagulated)	0·168	0·17*	0·195
Edestin (native)	0·14	0·17	—

* Interpolated from data given by Barker (1933).

2—The hydration term w is a minimum value, as stated in §5, moreover this minimum value is obtained in concentrated solutions where the vapour pressure or the activity of water is low. Unless there is some unknown compensating factor, the values of w should represent the minimum hydration for the proteins dissolved in dilute salt solutions, where the activity of water is high.

3—The evidence for hydration is obtained not only with concentrated solutions of electrolytes but also with dilute (0·01 M.) solutions containing sucrose.

In the ultra-centrifugal measurements of Svedberg (1930), the observations have been interpreted on the assumption that the protein molecules existing in solution are anhydrous. The observations recorded above afford evidence in favour of hydration which is of sufficient weight to

justify a number of recalculations. In the case of haemoglobin in sodium phosphate buffer of p_H 6 the density of the crystal (1·225) corresponds to a hydration value of 0·344.

If the molecular weight of the dry protein be 67,000, the molecular weight of the hydrate is 90,050. The molecular volume is increased by 45·8% and the molecular radius is increased by 13·4%. Svedberg (1930) has defined and calculated a dissymmetry number symbolized $f f_0$ which serves to measure the deviation of molecules from the spherical form. For haemoglobin $f/f_0 = 1 \cdot 25$ if it be assumed that the molecule is anhydrous. A recalculation using the value $w = 0 \cdot 344$ diminishes $f f_0$ to 1·10. The allowance for hydration reduces the apparent deviation from a spherical form.

Summary

A method is described for the determination of the density of protein crystals in aqueous media. Measurements have been made on crystals of edestin, egg albumin, horse serum albumin, haemoglobin, also on amorphous euglobulin, and on egg albumin and haemoglobin after heat coagulation.

The observed density of protein crystals is a function of the composition and hydrogen ion concentration of the medium used for the measurement of the density. It is probable that the protein crystals are highly permeable and reach a state of dynamic equilibrium with all of the components of the medium.

In all the cases examined, the density of the crystals is definitely less than the apparent density of the anhydrous protein in aqueous solution. Evidence that this diminution in density is due to the presence of water of hydration of the crystal is discussed in the text.

The plant globulin edestin has a considerably higher crystal density than any of the animal proteins investigated.

The density of heat coagulated proteins is only slightly greater than that of the corresponding protein crystals.

The presence of sucrose in the medium causes an increase in the value obtained for crystal density.

Formulae have been derived for the correlation of the observed density of protein crystals and their chemical composition, in order to test the hypothesis that the relatively low density of the protein crystal is due to hydration.

Determinations of the partial specific volumes of proteins in the crystalline form show that it is improbable that the relatively low density of

protein crystals is due to a contraction in volume when the crystals are dissolved.

Evidence as to the hydration of protein molecules in solution and the effect of such water of hydration on the dissymmetry number of the protein is discussed in the text.

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A Quantitative Study of the Action of Ultra-Violet Light on Bacteria

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[PLATE 13]

INTRODUCTION

The experiments here recorded were made between 1929 and 1932. The work was then unfortunately interrupted, and since the death of the senior author the material has been prepared for publication by M. C.-R.

Experiments on different species of infusoria carried out by Dreyer (1903), using the carbon arc as a source of light and different filters, showed that, although the greatest lethal effect was always obtained in that part of the spectrum which passes through glass, the relative sensitivity of different infusoria varied considerably in different parts of the spectrum. Sonne (1927) found that the line 2800 A of the mercury spectrum exhibited the greatest lethal effect on paramaecia, while in the case of haemolysis the most effective line was 2400 A. Hausser and Vahle (1921) found the erythema-producing maximum at 2970 A. Thus, the different spectral lines appeared to show some selectivity in their biological activity, and in view of this it was of interest to investigate the effect of these lines on different species of bacteria. Also, it would be of interest to investigate the changes in sensitivity to light induced by treating the organisms with erythrosin. As it was possible that the differences in some cases might be very slight, it was essential to use a technique which would reduce the experimental error as much as possible.

TECHNIQUE

An agar layer of uniform thickness of 1·15 mm on a glass plate 107 × 82 mm was prepared. A stock agar of 4% veal peptone bouillon agar p_H 7·2 to 7·6 was liquefied until quite limpid and, using graduated test-tubes, 4·2 cc of the agar were added to 4·2 cc of hot sterile bouillon, or, for sensitized plates, to 4·2 cc of a hot 1/8000 solution of erythrosin in distilled water, carefully mixed, and poured on to the prepared glass

plate. The details of the technique have been previously described (Dreyer and Campbell-Renton, 1933).

The plates were inoculated as follows: A 20-hour bouillon culture of the organism was mixed with an equal volume of sterile distilled water, or, in the case of the sensitized plates, with an equal volume of a 1/8000 solution of erythrosin in distilled water. Holding the plate by one corner and tilting it slightly downwards over a saucepan of water, 6 to 10 cc of the culture were poured over the entire agar surface and held in this position for a few seconds to drain off the excess fluid. The cultures when diluted contained approximately 400 million organisms per cc and the layer of culture on the plates about 50,000 to 100,000 organisms per sq mm. The variation in different cultures was not considerable. The plain cultures were poured on to the plain plates and the sensitized on to the sensitized—making the final concentration of erythrosin in the latter 1/16000.

With the sensitized plates great care was taken to expose the bacteria and erythrosin as little as possible to daylight.

After inoculation, to allow evaporation of superfluous moisture and prevent the inoculum from running, the plates were put in the incubator at 37° C for one hour before exposure in the spectrograph.

Exposure of Inoculated Plates

The source of light was a vacuum type quartz-mercury vapour arc lamp made by the Hewittic Electric Company. It was run off the 100-volt D.C. mains with a resistance in series which reduced the voltage across the lamp to 80 volts. At this voltage the current was 4 to 4·5 ampères. A choke of about 0·2 Henries was also used in series and kept the lamp running steadily at red heat. Mercury pools formed both anode and cathode electrodes. The length of the arc was 35–40 mm and the bore of the arc tube 16–17 mm.

The spectrograph was of a type made by Bellingham and Stanley and adapted to allow the plates to be exposed face downwards in a horizontal position. It was fitted with a 60° quartz prism. The focal length: aperture ratio of the collimator was about 10, that on the spectrum side of the prism varying from a lesser to a greater value. The effective prism aperture was about 2 inches, and the total spectrum about 9 inches long. Attached to the spectrograph was a plate-holder, designed to take photographic quarter-plates which held the agar plate in position.

The plate-holder could be set in any position covering a wave-length from 6000 to 2000 A. A constant aperture was used in comparative

experiments. The width and length of the slit were adjustable, but a slit width of about 1 mm which gave lines about 2 mm wide on the agar plates was found to be the most suitable. The plate could be moved in two directions perpendicular to each other by a rack and pinion movement, and fixed in a definite position as required. It was possible to get 8 to 10 exposures on each plate by this means, and in our experiments we set the plate-holder to cover a wave-length of from either 5780 to 2893 A, or 3132 to 2300 A.

Fig. 1, Plate 13, shows a plate of *B. coli* (Lister) exposed to the spectrum 3132 to 2300 A, the times of exposures being 10, 15, 25, and 40 seconds, 1, 1·5, 2, 3, 5, and 8 minutes respectively.

The back of the plate was covered with a piece of thin black cardboard to prevent admission of light from any outside source, and all the exposures were made at room temperature and timed with a stop-watch.

To investigate the effect of any selected wave-length—eliminating the rest of the spectrum—we used a " spot " apparatus designed by Jeffree (1936, *a*). By this apparatus, a beam of light of any desired wave-length could be projected on to the plate where it formed a " spot " about 3 mm in diameter. A long series of exposures of varying times could thus be made on the same plate. Fig. 2, Plate 13, shows a culture of *B. typhosus* (Hopkins) exposed in this way for varying times to the line 2655 A.

Reading of Exposed Plates

After exposure the plates were incubated for 22 hours at 37° C in a moist chamber, and the amount of growth in any given line or " spot " was then determined by means of a special apparatus designed by Jeffree (1936, *b*) and made by S. Bush in this Department. Briefly, this apparatus —which will hereafter be called the " reading machine "—measured the opalescence of solid or liquid layers by means of the photoelectric effect of light scattered from them. A beam of light, passing obliquely upward through the plate could be focussed on to the bacterial growth on the surface of the agar in an area slightly smaller than the image of the spectral line or " spot ".

Variations in illumination were compensated by balancing the scattered light against that transmitted directly through the layer, using two similar photoelectric cells. The cell cathodes were potassium hydride gas-filled and sensitive to blue light. The photoelectric currents were measured by a Lindemann electrometer—a greatly magnified image of its fibre being projected upon a scale. The scale was calibrated directly in terms of optical wedges composed of white paint in Canada balsam, and, when

compared with suspensions of dead bacteria in water in thin horizontal cells, it was found to be approximately accurate. Readings were reproducible even after long intervals, and fluctuations in the illumination, or coloured screens of reasonable density placed between the plate and the light, had very little effect on the readings obtained.

Calculations of Readings Obtained

All rates of bactericidal action were expressed in percentages of the readings recorded on the "reading machine" for 22 hours' growth in the unexposed parts of the plate. These "ground" readings were taken above and below the exposed area—but far enough away to avoid any increased growth at the edges of the area—and averaged. Similarly, two readings in each exposed line or "spot" were also averaged. The reading for "sterile", which represents the opalescence of the agar plate plus the original inoculum which had not grown at all, owing to the effect of a maximum exposure, was subtracted from the "ground" reading and the result taken as 100% of bacterial growth. The "sterile" areas were always examined subsequently with a magnifying lens to make certain that there were no undetected colonies.

Thus, if the average "ground" reading was 80, the exposed area 60, and the "sterile" 10, 80 minus 10 equals 70 was taken as 100% of bacterial growth. Accordingly, the reading of the exposed area being 60, the reading for "sterile" (10) must also be subtracted from this figure, and the figure obtained was 50, which represents 71·5% growth—which must be the result when the figure 70 represents 100%.

Table I shows an example of actual readings obtained in a duplicate experiment on the same plate with *B paratyphosus B.* (Kasauli) 2655 A "spot".

Drawing of Curves

For each organism a curve was drawn in which the percentages of growth after exposure to some selected wave-length for varying times were plotted against the logarithms of the times of exposure. A number of experiments was made with each organism and the composite results were used in drawing the "smoothed" curve. The abscissae of the points on the smoothed curve corresponding to the measured percentage of growth were read off and their anti-logarithms found. These were then replotted against the percentages of growth to give what is conveniently called a "time curve".

TABLE I—EXAMPLE OF ACTUAL READINGS

Experiment December 3, 1931. Duplicate on same plate. *B. paratyphosus B* (Kasauli). Lamp (3) 4·2 sec and 80 volts—end of experiment 4·0 sec and 81 volts. Sterile = 20·9. A 2655 " spot ".

Time sec	(1) Reading	(1) Ground	(1) Growth %	(2) Reading	(2) Ground	(2) Growth %
3·0	53·3		100	54·2		100
		53·3			54·2	
	52·7			54·0		
4·0	53·0		99·3	54·2		98·5
		53·5			55·0	
	52·3			52·7		
5·0	52·5		97·5	52·9		96·0
		53·0			53·5	
	51·9			50·5		
6·5	51·9	54·0	94·8	50·8	53·0	92·1
		52·5				
	50·5					
8·0	50·0		91·3	—	—	—
		53·5				
	50·0			49·6	54·0	
10·0	50·0		87·7	50·0		86·9
		54·5			54·5	
	48·4			—		
12·5	49·0					
		55·0	82·2		54·5	—
	46·5			47·0		
16·0	47·6		76·6	47·2		78·7
		55·0			54·0	
	45·9			45·1		
20·0	47·0		75·0	46·0		73·3
		55·0			55·0	
	44·3			44·1		
25·0	45·1		69·7	45·2		69·6
		55·0			—	
	41·1			42·1		
30·0	42·8		62·3	43·5		64·1
		54·5			55·0	
	39·5			40·5		
35·0	41·2		58·3	42·0		60·5
		54·0			54·0	
	39·2			38·6		
40·0	41·2	55·0	57·3	39·7	54·0	55·3
		54·0			54·0	
	36·2			36·0		

TABLE I—(continued)

Time sec	(1) Reading	(1) Ground	(1) Growth %	(2) Reading	(2) Ground	(2) Growth %
50·0	37·3		47·8	37·7		48·2
		54·0			54·0	
	33·8					
65·0	35·1		40·7	—		—
min		54·5			54·0	
(1) 18	*20·9S			20·9S		
(2) 9	21·0		0	‡21·0		0
		54·5			55·0	
	31·0			31·0		
80·0	32·1		31·5	32·9		32·3
		55·0			55·0	
	27·5			28·8		
100·0	30·0		23·4	30·0		24·9
		54·0			55·0	
	24·6			26·7		
125·0	26·0		13·3	27·2		17·8
sec		54·0			54·5	
(1) 45·0	†35·9			23·6		
min						
(2) 4·0	37·6		48·7	§23·6		7·88
		53·0			56·0	

S = Sterile. * 18 min. † 45·0 sec. ‡ 9 min. § 4·0 min.

The shape of the logarithm curve for any given microbe was always consistent, irrespective of the wave-length of light used. Sensitization of the organisms with erythrosin before exposure to light did not alter the shape of the curve. The curves for different microbes were not parallel, however, and in fig. 3 are shown actual duplicate observations with three different organisms exposed to the line 2655 A. In some cases, even organisms which were serologically indistinguishable showed marked differences in their susceptibility to light, and their curves were of different shape. In other words, the relative sensitivities of different organisms to the same wave-length were not constant with varying times of exposure. These findings are contrary to those of Wyckoff (1932), who exposed cultures of *B. coli* and *S. Aertrycke* to the lines 3132 and 2803 A and found the shape of their curves from 95 to 10% survivors to be identical. In order to compare the bactericidal effect of different spectral lines on the same organism, or the effect of the same spectral line under different experimental conditions, we used the time curve of that particular organism as an arbitrary standard. The times of exposure for each observed

percentage growth were divided by the times read off from the time curve corresponding to the same percentage growth. The quotients thus obtained were averaged. The average variation among the quotients

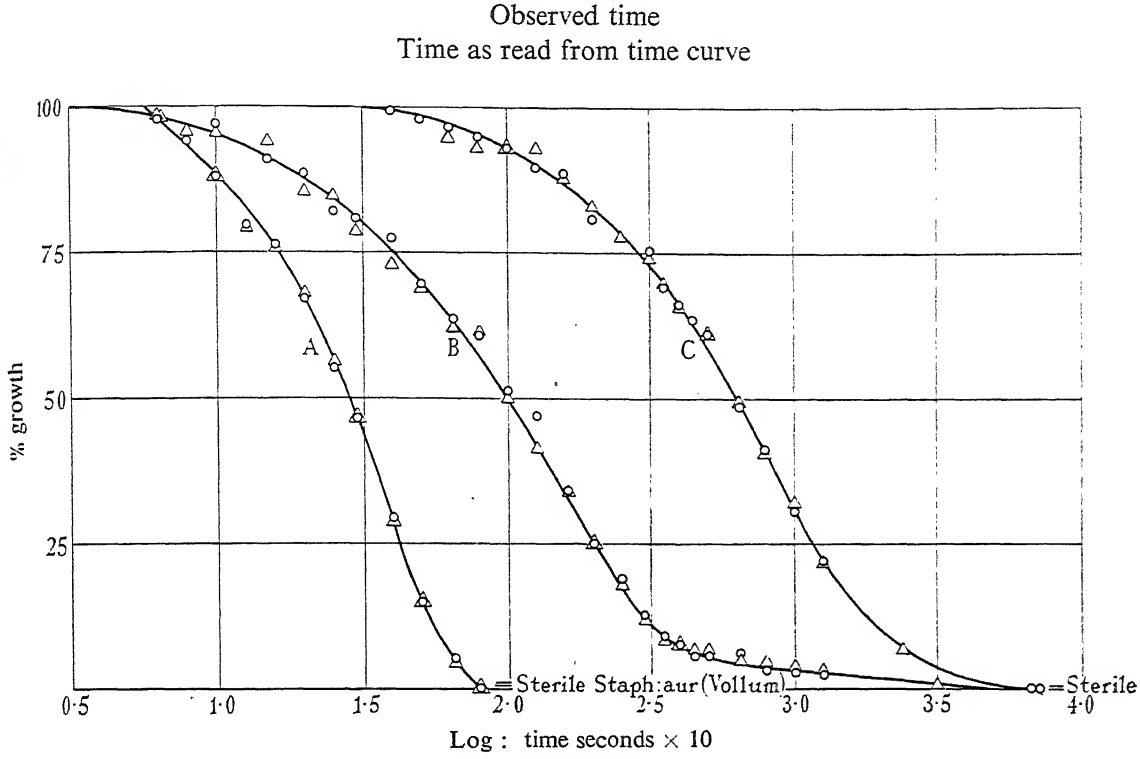

FIG. 3—○ = reading 1 duplicate experiments; △ = reading 2 "spot" 2655 A. (A) *Staph. aureus* (Vollum) times of exposure 2, 2·5, 3, 4, 5, 6·5, 8, 10, 12·5, 16, 20, 25, 30, 40, 50, 65, 80, 100, and 125 seconds. (B) *B. dysenteriae Flexner* W (Logan) times of exposure 0·5, 1, 1·5, 2, 2·5, 3, 4, 5, 6·5, 8, 10, 12·5, 16, 20, 25, 30, 35, 40, 50, 65, 80, 100, and 125 seconds, and 5 and 12 minutes. (C) *B. paratyphosus B* (Topley) times of exposure 0·5, 1, 1·5, 2, 2·5, 3, 4, 5, 6·5, 8, 10, 12·5, 16, 20, 25, 30, 35, 40, 50, 65, 80, 100, and 125 seconds, and 4, 9, and 17·5 minutes.

in the case of a given microbe corresponded to a percentage standard deviation of 5 to 9%.

The average quotient served as an index of the bactericidal effect on the same organism.

To compare the bactericidal effect of the same spectral line on different organisms, whose "time curves" were not parallel, it appeared that the best point of comparison was in that part of the curve which most nearly approached a straight line. This was found to be in that part of the curve

corresponding to 40% growth. However, in many experiments (*e.g.*, where weakly bactericidal lines were used) 40% growth did not come into the range of observations. In order to have a standard procedure which could be applied in all cases, we adopted the following device: in each experiment the average quotient obtained as described above was divided into the time corresponding to 40% growth on the appropriate " time curve ", and the figure thus obtained, which we have called " K ", has been used to express the relationship between different organisms. In Table II is given an example of three different strains of *B. paratyphosus B* exposed to the " spot " line 2655 A. The curves of these strains were quite parallel and, applying the same " time curve " in each case, it will be seen that the sensitivity of the three strains is different, the average quotients being 1·019, 2·190, and 1·590. The time corresponding to 40% growth on the " time curve" for this organism is 36·31 seconds, and the Ks for the three strains are therefore 35·636, 16·58, and 22·84 respectively. The Ks for each microbe where their curves were not alike were, of course, always calculated, each from its own " time curve ", at the point corresponding to 40% growth.

As has been mentioned, a number of microbes which were otherwise indistinguishable, had differently shaped curves. In Table III are given the figures of the curves determined for four strains of Staphylococcus, and it will be seen that the three strains of *Staphylococcus aureus* are all different.

If one subtracts the logarithms of the times found for *Staph. aureus* (B. B.) from those for *Staph. aureus* (Vollum) for the same percentages of growth, there is a definite periodic decline from +0·407 at 100% growth to +0·076 at 10% growth. In the case of subtracting those of *Staph. aureus* (B. B.) from *Staph. aureus* (Eye 4) there is an increase from +0·239 at 100% growth to +0·441 at 65% growth, and then a periodic decrease to +0·334 at 5%.

On the other hand, we found that *Staph. aureus* (M. J.), which was freshly isolated, and *Staph. aureus* (Krueger), an old stock culture, had curves identical with *Staph. aureus* (B. B.), and that two other strains called *Staph. aureus* (Yellow) and *Staph. aureus* (White) were identical.

Fig. 4 shows the curves calculated for *B. dysenteriae V*, *W*, *Y*, and *X*, and for *B. dysenteriae Shiga* (Lister) which all differ from each other. " Rough " and " smooth " variants of *B. dysenteriae Shiga* (Lister) were alike, as also was *B. dysenteriae Shiga* (C. R. M.) " smooth ".

Fig. 5 includes two strains of *Salmonella Aertrycke*—(Glasgow) and (Mutton)—with different curves.

Fig. 6 shows two strains of *B. paratyphosus A* with quite different curves,

TABLE II

B. paratyphosus (B . HB_2)				*B. paratyphosus B* (Topley)			*B. paratyphosus B* (Kasauli)		
Time sec	% growth	Time curve	Time observed / Time curve	% growth	Time curve	Time observed / Time curve	% growth	Time curve	Time observed / Time curve
2·5	98·5	2·6	0·962	—	—	—	—	—	—
3·0	97·4	3·1	0·969	—	—	—	—	—	—
4·0	96·4	3·5	1·140	—	—	—	99·3	2·43	1·646
5·0	92·8	5·0	1·000	—	—	—	97·5	3·09	1·618
6·5	89·7	6·3	1·031	—	—	—	94·8	4·20	1·548
8·0	88·0	7·0	1·142	—	—	—	91·3	5·60	1·429
10·0	83·5	9·0	1·111	93·0	4·9	2·04	87·7	7·08	1·413
12·5	78·4	11·4	1·096	89·8	6·2	2·02	82·2	9·10	1·312
16·0	67·5	16·9	0·947	88·7	6·8	2·35	76·6	12·20	1·307
20·0	59·7	21·3	0·939	81·0	10·1	1·98	75·0	13·10	1·527
25·0	53·2	25·9	0·965	—	—	—	69·7	15·80	1·582
30·0	46·6	31·0	0·968	75·4	12·8	2·34	62·3	17·20	1·744
35·0	39·9	36·0	0·973	69·3	16·0	2·19	58·3	22·52	1·554
40·0	35·4	40·5	0·988	66·1	17·7	2·26	57·3	23·05	1·736
50·0	29·4	47·5	1·051	61·2	20·6	2·43	47·8	30·00	1·667
65·0	20·3	63·0	1·031	49·0	29·3	2·22	40·7	35·70	1·738
80·0	15·05	75·0	1·068	41·0	35·5	2·25	31·5	44·75	1·788
100·0	9·95	98·0	1·020	30·9	45·5	2·20	23·4	56·5	1·770
125·0	5·82	129·0	0·970	22·1	58·7	2·13	13·3	73·25	1·707
45·0	31·50	44·8	1·005	63·7	21·8	2·06	48·7	29·5	1·525
		Average =	1·019		Average =	2·190		Average =	1·590
		6% =	6·05%		6% =	6·10%		6% =	9·276%

40% growth on curve = 36·31 seconds
therefore

$\frac{36 \cdot 31}{1 \cdot 019}$ = K 35·636 seconds

$\frac{36 \cdot 31}{2 \cdot 19}$ = K 16·58 seconds

$\frac{36 \cdot 31}{1 \cdot 59}$ = K 22·84 seconds

TABLE III—SMOOTHED CURVES. LAMP (3)

% growth	(1) *Staphylococcus albus* (Pasteur) Log T sec × 10	(2) *Staphylococcus aureus* (Eye 4) Log T sec × 10	(3) *Staphylococcus aureus* (Vollum) Log T sec × 10	(4) *Staphylococcus aureus* (B. B.) Log T sec × 10	(3) — (4)	(2) — (4)
100	0·955	1·580	1·748	1·341	+0·407	+0·239
95	1·128	1·867	1·869	1·505	+0·364	+0·362
90	1·280	2·057	1·976	1·650	+0·326	+0·407
85	1·408	2·198	2·062	1·775	+0·287	+0·423
80	1·520	2·303	2·144	1·876	+0·268	+0·427
75	1·618	2·397	2·213	1·960	+0·253	+0·437
70	1·710	2·473	2·272	2·033	+0·239	+0·440
65	1·800	2·536	2·323	2·095	+0·228	+0·441
60	1·883	2·585	2·371	2·152	+0·219	+0·433
55	1·962	2·631	2·415	2·208	+0·207	+0·423
50	2·025	2·675	2·455	2·263	+0·192	+0·412
45	2·095	2·715	2·492	2·320	+0·172	+0·395
40	2·163	2·756	2·529	2·372	+0·157	+0·384
35	2·230	2·795	2·566	2·419	+0·147	+0·376
30	2·300	2·835	2·597	2·472	+0·125	+0·363
25	2·355	2·877	2·628	2·519	+0·109	+0·358
20	2·408	2·918	2·664	2·565	+0·099	+0·353
15	2·460	2·961	2·700	2·612	+0·088	+0·349
10	2·515	3·007	2·747	2·671	+0·076	+0·336
5	2·581	3·068	2·810	2·734	+0·076	+0·334
0	2·860	3·215	2·910	2·829	+0·081	+0·386

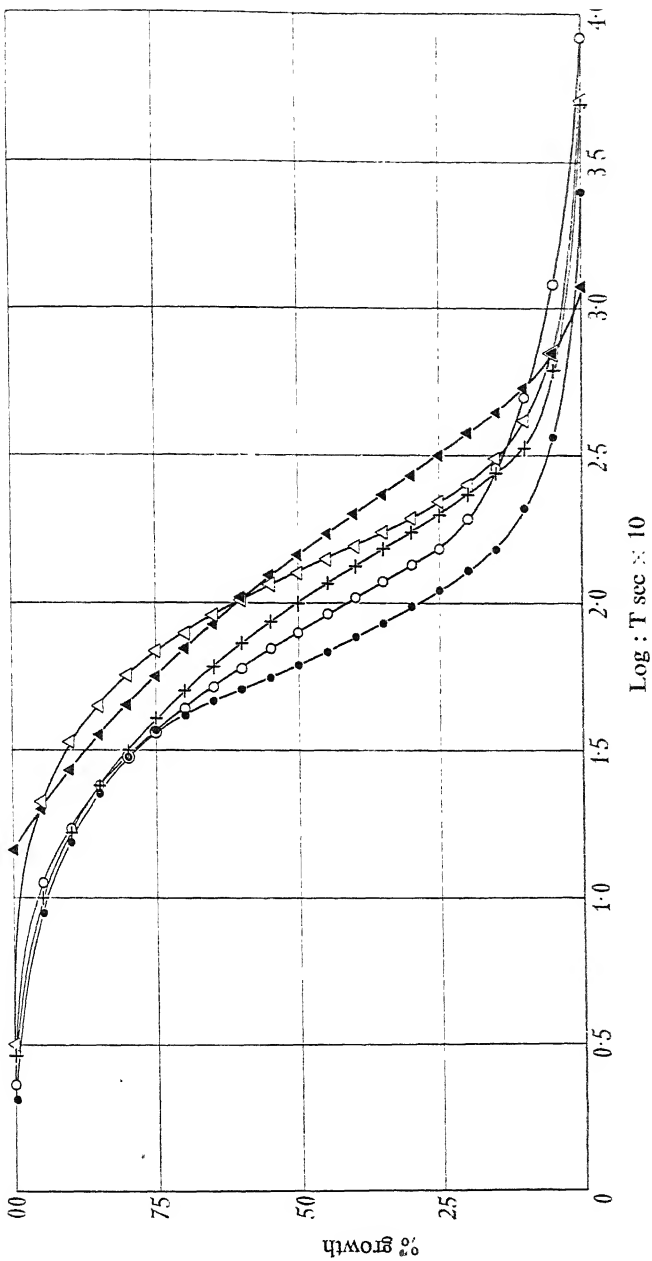

FIG. 4—Smoothed curves—"Spot" 2655 A. Lamp (3). 2·8 to 3·45 seconds and 81 to 84 volts throughout. ○ = *B. dysenteriae Flexner V* (Mott); + = *B. dysenteriae Flexner W* (Logan); △ = *B. dysenteriae Flexner Y* (Ledingham); ● = *B. dysenteriae Flexner X* (Toner); ▲ = *B. dysenteriae Shiga* (Lister).

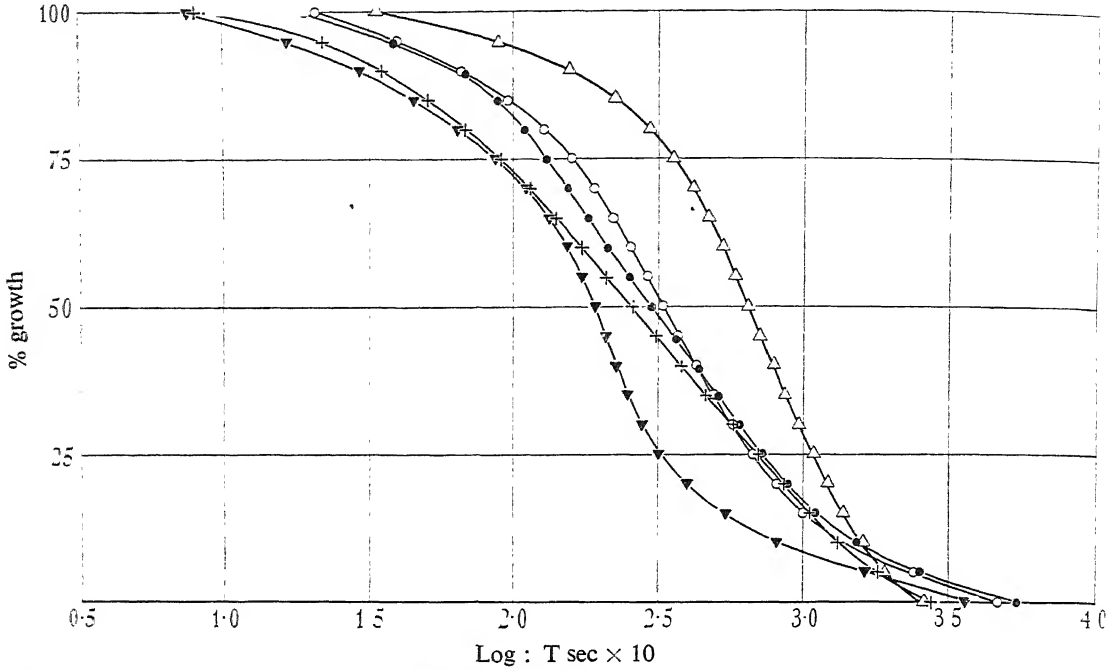

FIG. 5—Smoothed curves—" Soot " 2655 A. Lamp (3). 3·0 to 4·0 seconds and 81 to 84 volts throughout. ○ = *S. Aertrycke* (Glasgow); ● = *S. Aertrycke* (Newport); △ = *S. Aertrycke* (Mutton); + = *B. Gaertner* (Dennie); ▼ = *Salmonella* (Reading).

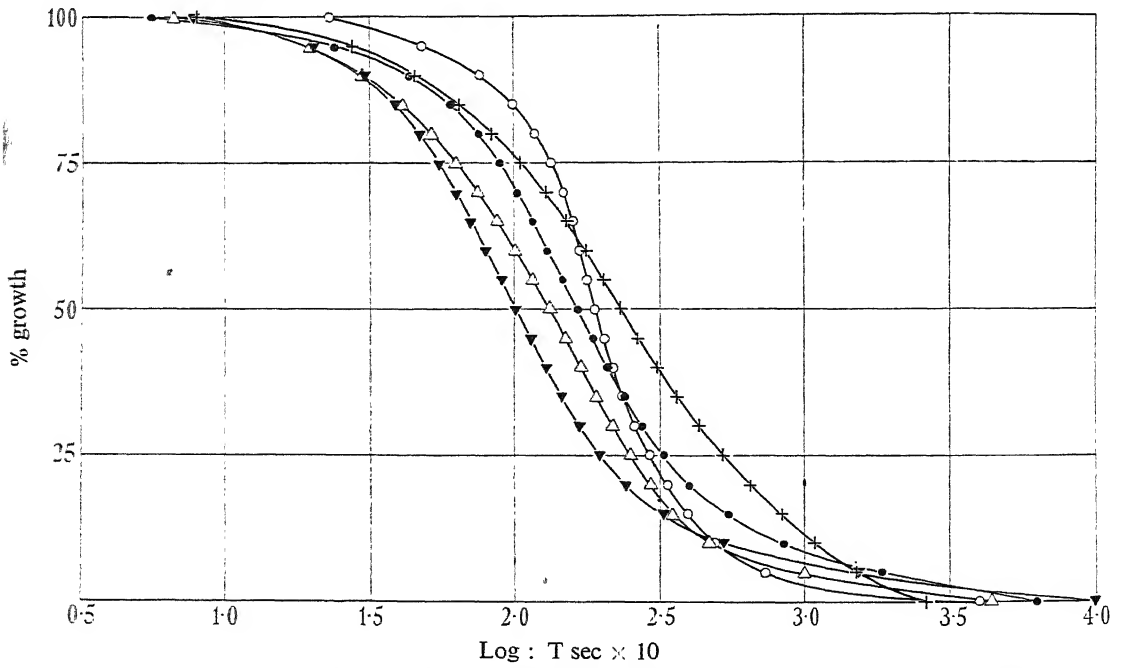

FIG. 6—Smoothed curves—" Spot " 2655 A. Lamp (3). 3·8 to 4·2 seconds throughout. ● = *English Hog Cholera*; ○ = *V. Metchnikoff*; △ = *B. paratyphosus A* (Schottmuller); + = *B. paratyphosus A* (Hadarsah); ▼ = *B. typhosus* (Rawlins).

but *B. typhosus* (Rawlins) was identical with *B. typhosus* (901), "smooth O" (901), "rough O" (901), "smooth H" (901), "rough H", and *B. typhosus* (Hopkins).

Fig. 7 shows the curves calculated for two strains of *B. coli*—*B. coli* (R. 29) containing a greater proportion of resistant organisms than *B. coli* (Lister)—*B. paratyphosus B* (H. B. 2) which has been shown to be identical with (Kausali) and (Topley); *C. hofmannii*, and *yeast* (Danish), where a large proportion of the cells were killed in the shorter exposures, but longer times were required to kill the remaining resistant cells.

Experimental Variation in Result.

(1) *Deterioration of the Lamp*—By continued use the intensity of the light not only became weaker, but the different spectral lines were affected in varying degrees. Although there was no change in the shape of the curves for the bacteria, the former explains the longer times required to obtain a given bactericidal result, while the latter partially explains the change in the relative activity of different lines. The difference in intensity in different lines of the spectrum found in the same lamp tested at various intervals of time were very slight, and did not appear to be consistent. Three lamps were used during the course of our experiments, and in Table IV is shown an average of three readings with lamp 2, and three readings with lamp 3, taken during a period of about 6 months, and the difference in intensities found in the spectral lines with the two lamps.

These measurements of intensity were made with a "spectro-integrator" designed by Jeffree (1936, *c*). In this instrument a photoelectric spectrophotometer was interposed between the two lenses of the condenser system of the spectrograph. Either the whole spectrum or any selected wave-length to be measured was passed to a sodium-quartz photoelectric cell, and the charge of an air dielectric condenser was recorded by a Lindemann electrometer. The eye-piece scale, on which the movement of the electrometer fibre was recorded, was calibrated in terms of absolute energy units received during a given exposure. For our measurements of the intensity of the total spectrum and the various lines, we took the time in seconds which the fibre took to record 16×10^{-5} ergs per sq cm.

This instrument was employed at the beginning, middle, and end of every experiment, and we were thus able to check any fluctuations in the lamp.

(2) *Variation in the p_H of the Bouillon*—The optimum p_H of the inoculum was found to be between 7·2 and 7·6, and if the p_H was increased or

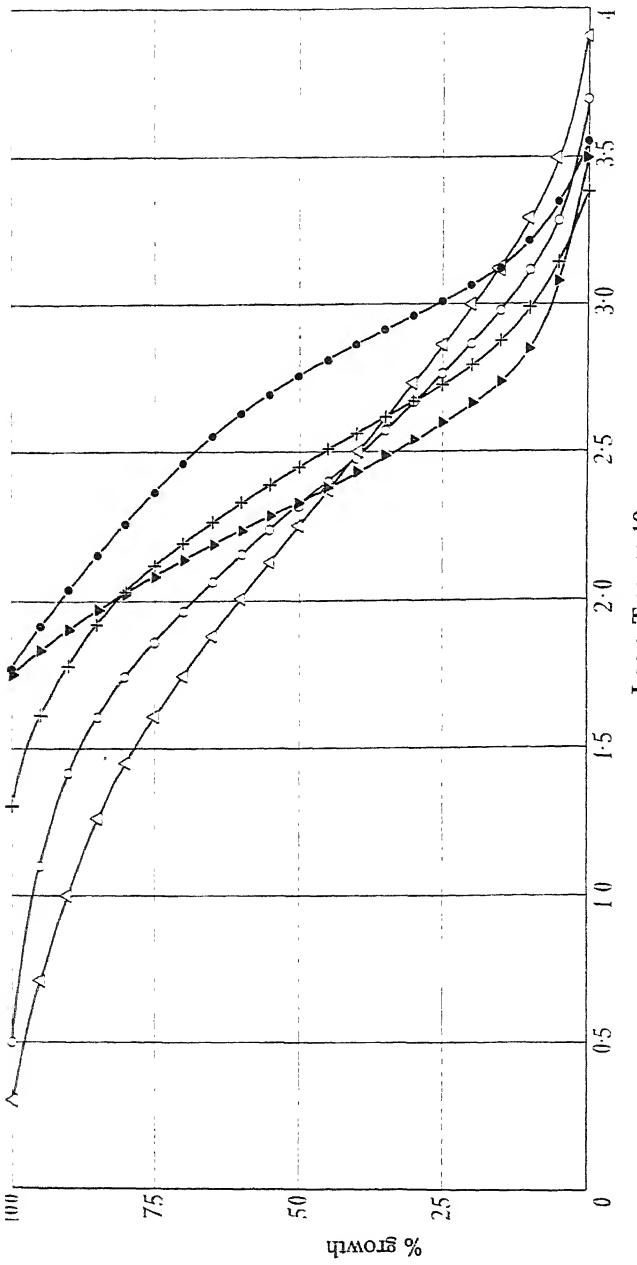

FIG. 7—Smoothed curves. "Spot" 2655 A. Lamp (3). 3·4 to 4·0 seconds and 79 to 82 volts throughout. ○ = *B. coli* (Lister); △ = *B. coli* (R. 29); + = *B paratyphosus B* (H. B. 2); ● = *C. Hofmanni*; ▼ = *Yeast* (Danish).

decreased the microbes became more sensitive. This was more marked when the p_H was increased. In an experiment with *Staph. aureus* (Vollum) grown in bouillons of p_H 8·5 and 6·5, the alkaline culture was 12·7% more sensitive than the acid.

TABLE IV—TIMES IN SECONDS AT 16 ERGS

A	Lamp (2) Average of experiments 22/4/31, 12/5/31, 21/5/31	Lamp (3) Average of experiments 16/6/31, 29/6/31, 28/10/31	Lamp (2) / Lamp (3)
4358	—	36·66	—
4047	—	45·43	—
3655	18·35	13·58	1·35
3342	189·75	132·34	1·43
3132	36·74	22·72	1·62
3022	54·48	31·31	1·74
2967	80·94	59·40	1·36
2925	—	91·18	—
2893	247·15	126·77	1·95
2804	182·09	98·14	1·85
2760	218·68	158·13	1·38
2700	166·00	74·68	2·22
2655	115·70	56·79	2·04
2580	—	—	—
2536	98·24	55·26	1·78
2482	270·15	121·60	2·22
2400	311·00	143·16	2·17
2378	364·05	166·70	2·18
2350	472·10	195·90	2·41
2320	472·16	194·15	2·43
2300	486·37	198·30	2·45

(3) *Density of Cultures*—Within wide limits the density of the inoculum appeared to have no effect on the time of exposure required to reach a given bactericidal effect, and thus there appeared to be no loss of sensitivity due to one microbe "screening" another. A culture of *B. dys. Flexner X* had an average "ground" reading (after, of course, subtracting the reading for "sterile") of 28·5 on the "reading machine", and its K for 40% growth was 9·33 seconds, while the same bacterium exposed to the same spectral line (2655 A) nine days later had an average "ground" reading of 54·5 and a K of 7·76 seconds. Similarly a culture of *Staph. aureus* (B. B.), diluted 1 in 3 before inoculation, was only 0·54% more sensitive in four spectral lines than the undiluted control of the same culture exposed to the same lines on the same day; whereas in another similar experiment, using a culture of *Staph. aureus* (M. J.)

diluted 1 in 10, the diluted culture was 0·11% more resistant than the control, which was well within the limits of experimental error.

(4) *Concentration of Agar*—In order to determine what effect the concentration of agar in the plates had on the sensitivity of the bacteria to light, we exposed two plates inoculated with the same culture of *Staph. aureus* (Vollum) on the same day, one having a 1·5% and the other a 4·0% concentration of agar. We found that the microbes on the plate with 1·5% agar were 65% more sensitive than those on the 4% concentration, but the shape of the curves remained identical.

Apart from the factors mentioned, there may, of course, be unexplained causes resulting in slight variations in the results obtained at different times with the same microbe, although the curves remain the same. Thus, it must be emphasized that only experiments done on the same day —and even on the same plate—are strictly comparable.

The Effect of Reducing the Intensity of the Light by Screening

To investigate the relation of the intensity of light to the bactericidal power, the following experiments were carried out. The reduction of the intensity of light passing through the optical parts of the spectrograph was effected as follows: three metal screens with slits of different widths were placed in such a manner that the light, after passing through them, was properly diffused, and so constructed that screen 1 reduced the intensity to half, screen 2 to a quarter, and screen 3 to an eighth of the intensity obtained without screening. If the bactericidal effect, both absolutely and relatively, in different lines of the spectrum followed the ordinary laws, the times required to obtain a given effect would be inversely proportional to the intensity of the light. Our findings with several species of bacteria showed this actually to be so. Thus we found that, within the errors of the screens, the times required to obtain the same effect were respectively, two, four, and eight times as long as without the screens. That this law only holds good over a certain range of intensities is obvious, as we know that minute amounts of light have no apparent bactericidal effect. Our findings, however, were consistent not only where wave-lengths of from 3132 to 2300 A were used, but also for the wave-lengths from 5780 to 2893 A when bacteria sensitized with erythrosin were employed.

Relative Bactericidal Effect of the Different Spectral Lines

In agreement with Gates (1930) and other workers, we found that, with all the species of bacteria we tested, the line 2655 A was the most bactericidal. The next in order of effectiveness were the lines 2536, 2804, 2482, and 2700 A. The lines 2760 and 2580 A were about equal, and 2893 A was, except for *B. coli* (Lister), stronger than 2400 A.

The lines 2967 and 2378, 2300 and 2350, and 2320 and 3022 A respectively, appeared to vary in relative efficacy according to the microbes employed. In Table V are shown examples of different bacteria exposed to the spectrum. The figures, which represent 40% growth, were, for purposes of comparison, divided by the times found in each instance for 40% growth in the line 2655 A—the actual times in minutes for this line are also given in the table. The figures given were obtained by averaging the results of three to six experiments with each microbe exposed on different occasions. It will be seen that the relative sensitivity with different microbes appears to vary considerably in certain spectral lines, which, even allowing for certain experimental errors and variations, seems to suggest some degree of selectivity. As will be seen, the greatest relative differences appear in the lines 3022, 2378, 2400, and 2300 A, while in the other lines the difference in values was less marked.

Effect of Disinfectants

In order to compare the effect of disinfectants on bacteria with that of light, we prepared plates as already described and, after inoculation, dried them for an hour. A series of different dilutions of the disinfectant employed was made, and with a platinum loop 3·6 mm in diameter, a loopful taken from each dilution and carefully put on the plate without touching the surface of the agar with the loop. During this process the plate was placed on a white card of the same size, on which several rows of circles, correctly spaced, had previously been drawn in red. The circles were visible through the agar, and a loopful of the dilution—which spreads over an area of about 7 mm in diameter—was placed on each circle. About ten dilutions in duplicate could be put on each plate in this manner. The plates were then incubated for 22 hours at 37° C in moist petri dishes, and the percentage of growth in the different dilutions read on the "reading machine" in the same manner as was done with the exposed plates. The percentage of growth was plotted against the logarithm of the reciprocal of the concentration of disinfectant,

TABLE V—RELATIVE BACTERICIDAL EFFECTS IN DIFFERENT LINES OF THE SPECTRUM. K FOR EACH SPECTRAL LINE DIVIDED BY K FOR 2655 A FOR EACH MICROBE

	Lamp (2)				Lamp (3)					
A	*Staph. aureus* (Yellow)	*B. coli* (Lister)	*C. Hofmannii*	*Staph. aureus* (Eye 4)	*Staph. aureus* (Vollum)	*Staph. aureus* (Krueger)	*Staph. aureus* (B. B.)	*Staph. aureus* (M. J.)	*Staph. albus* (Pasteur)	*B. dys. Shiga* (Lister)
3022	42·54	20·43	34·91	25·21	63·03	119·0	76·87	43·00	—	24·44
2967	23·64	8·71	13·78	11·27	31·86	19·10	22·90	16·45	24·09	11·73
2925	—	38·30	59·56	40·76	—	—	—	—	—	—
2893	11·33	5·91	7·30	6·47	14·94	9·86	14·00	8·56	15·55	8·90
2804	2·18	1·86	1·69	1·79	2·97	2·13	4·05	2·84	2·88	2·40
2760	10·30	5·69	6·92	5·84	11·06	9·67	6·12	10·53	14·95	8·83
2700	6·24	3·67	4·84	3·66	5·92	6·83	5·68	6·51	8·89	5·19
2655	*1·00*	*1·00*	*1·00*	*1·00*	*1·00*	*1·00*	*1·00*	*1·00*	*1·00*	*1·00*
2580	6·84	5·80	5·75	6·01	5·60	—	—	10·57	—	—
2536	1·21	1·18	1·38	1·26	1·02	1·67	1·93	1·94	2·06	1·49
2482	4·38	2·13	3·13	2·98	3·49	4·34	4·30	4·49	5·02	4·70
2400	15·67	5·80	8·74	9·60	15·41	24·99	20·38	17·10	26·00	16·01
2378	20·85	6·96	13·27	17·46	21·70	26·87	31·00	23·38	32·71	17·09
2350	34·09	16·82	17·11	23·70	34·12	43·00	—	—	—	35·30
2320	33·19	37·02	43·71	38·73	—	78·14	—	—	—	—
2300	22·27	22·12	22·27	21·50	26·86	—	—	—	78·89	41·05
2655 K =	*1·78*	*3·42*	*1·59*	*2·18*	*0·58*	*0·32*	*0·56*	*0·29*	*0·22*	*0·31* min

and fig. 8 shows the actual observations in seven experiments. Lysol was diluted with bouillon 1/20 to 1/640 and a duplicate series of loops put on plates of *B. coli* (Lister) and *Staph. aureus* (Vollum). In one experiment with *B. coli* there was a slight stimulation of growth in the

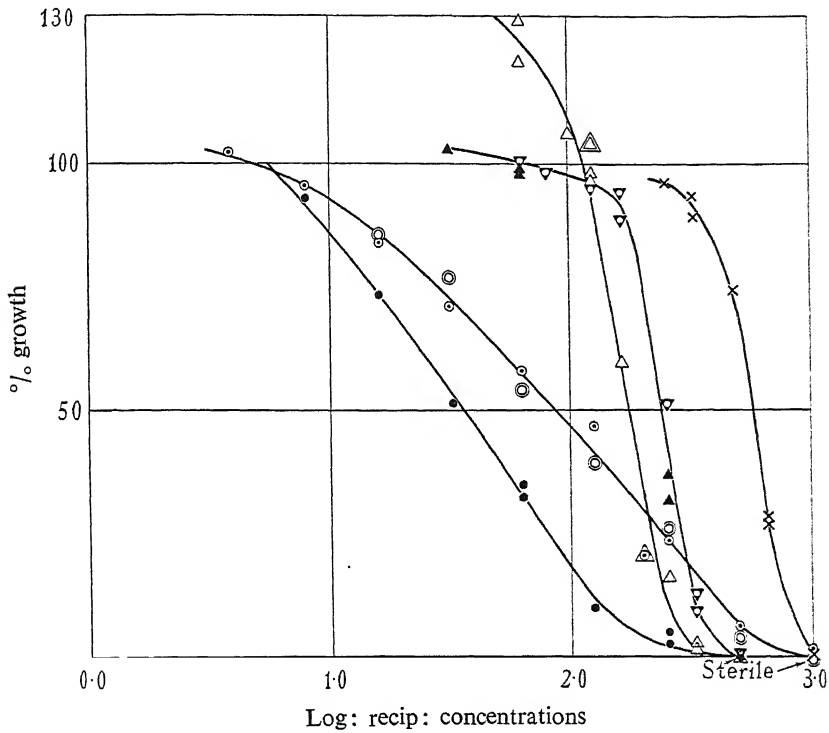

FIG. 8—● = 5% formalin on *B. coli* (Lister) dilutions in bouillon 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/5120, and 1/10240—the dilutions 1/80 and 1/320 being in duplicate. ○, ◎ = 5% formalin on *Staph. aureus* (Vollum). Two experiments made on different days. Dilutions 1/20 to 1/10240 as above. ▲ ▽, = 5% lysol on *B. coli* (Lister). Dilutions in bouillon 1/40, 1/80, 1/160, 1/320, and 1/640—the dilutions 1/320 and 1/640 being in duplicate. △ = 5% lysol on *Staph. aureus* (Vollum). Dilutions in bouillon 1/40, 1/60, 1/80, 1/100, 1/160, 1/200, and 1/320—the dilutions 1/60, 1/100, 1/160, and 1/320 being in duplicate. × = 5% phenol on *Staph. aureus* (Vollum). Dilutions in bouillon 1/20, 1/30, 1/40, 1/60, and 1/80—1/30 and 1/60 in duplicate.

higher dilutions—the increase in a 1/640 dilution of lysol being 2·5%—but with *Staph. aureus* (Vollum) the stimulation of growth in the higher dilutions was very apparent even to the naked eye, and the increase was 4·2% for a 1/160 dilution and 29% for a 1/320 dilution. Experiments with similar dilutions of phenol in bouillon did not appear to stimulate

growth so much, but from 100 to 0% growth, the curves were practically parallel with those of the lysol for both microbes.

The dilutions of formalin in bouillon were from 1/20 to 1/10240. In this case *Staph. aureus* (Vollum) was very slightly stimulated in the 1/5120 dilution, and it will be seen that the curves for *B. coli* and *Staph. aureus* (Vollum) are not parallel. Observations of two experiments done on different days are shown in the case of lysol on *Staph. aureus* (Vollum) and of formalin on *B. coli* (Lister), and it will be seen from the curves (fig. 4) that the results obtained with the same microbe were in both cases in close agreement.

The Effect of Sensitization

Table VI shows the effect of sensitization with 1/16000 erythrosin in the short wave-lengths on six species of microbe. The experiments were not all made on the same day, but in the case of each microbe a plain and a sensitized plate were inoculated with the same culture and exposed within an hour of each other. Readings were taken and the Ks for 40% growth calculated from the curves, and the K for the plain divided by the K for the sensitized in each spectral line. It will be seen that there is some difference in the relative sensitivity of the various microbes for the different lines in the sensitized as compared with the plain controls.

The greatest difference was found in the line 2655 A where *Staph. aureus* (Eye 4) was 1·27 times more resistant in the plain plate, while in the same line *Staph. aureus* (Krueger) was 0·43 times more sensitive. The next greatest discrepancy was in the line 2536 A where *Staph. aureus* (B. B.) was 0·45 times more resistant in the plain plate and *C. Hofmannii* 0·57 times more sensitive. That these findings were not dependent on the bactericidal strength of the line is indicated by the fact that in 2804 A—the next strongest line—*B. dysenteriae Shiga* (Lister) is only 0·28 times more resistant in the plain, and *Staph. aureus* (Vollum) 0·26 times more sensitive. On an average in the lines and microbes examined *Staph. aureus* (Eye 4) showed the least sensitization, where the average factor of plain divided by sensitized was 1·34, and *Staph. aureus* (Vollum) the most, with a factor of 0·897.

Sensitization in the Long Wave-lengths

The experiments in that part of the spectrum between 5780 and 3132 A were more conclusive than those in the short wave-lengths, as there appeared to be no effect—however long the exposure—in the unsensitized controls. The results of different concentrations of erythrosin were not

TABLE VI—EFFECT OF SENSITIZATION IN THE SHORT WAVE-LENGTHS WITH 1/16,000 ERYTHROSIN. K PLAIN DIVIDED BY K SENSITIZED

A	*C. Hofmannii*	*Staphylococcus aureus* (Eye 4)	*Staphylococcus aureus* (Vollum)	*Staphylococcus aureus* (B. B.)	*Staphylococcus aureus* (Krueger)	*B. dysenteriae Shiga* (Lister)
3022	1·07	0·70	1·24	1·35	1·80	0·79
2967	0·89	0·84	0·90	1·24	0·51	1·05
2925	0·56	—	0·85	—	—	—
2893	0·52	0·54	0·54	1·17	0·73	1·14
2804	0·95	1·16	0·74	1·15	0·77	1·28
2760	1·28	1·70	1·31	0·80	0·80	0·98
2700	1·17	1·51	1·11	1·25	0·88	0·95
2655	1·42	2·27	0·91	1·11	0·57	1·10
2580	1·41	1·57	0·68	—	—	—
2536	0·43	1·69	0·92	1·45	0·98	1·10
2482	—	1·41	0·67	1·19	0·87	1·51
2400	—	—	—	1·15	1·19	1·09
2378	—	—	—	1·17	1·26	1·04
2350	—	—	—	—	—	1·30
2320	—	—	—	—	—	—
2300	—	—	—	—	—	1·79

TABLE VII—EFFECT ON SENSITIZATION WITH 1/16000 ERYTHROSIN. K FOR EACH SPECTRAL LINE DIVIDED BY K FOR 2893 A FOR EACH MICROBE

A	*Staph. albus* (Pasteur)	*Staph. aureus* (B. B.)	*Staph. aureus* (Eye 4)	*Staph. aureus* (Vollum)	*Staph. aureus* (Yellow)	*Staph. aureus* (White)	*C. Hofmannii*	*B. dys. Flexner V* (45)	*B. dys. Flexner V* (37)
5780	7·97	3·70	—	7·69	17·62	12·41	2·03	2469·6	112·62
5461	2·83	0·89	1·64	5·36	5·02	3·51	1·44	245·74	29·96
4358	—	—	—	—	71·28	61·29	—	—	—
4047	—	—	—	—	112·25	111·80	—	—	—
3655	11·76	—	—	—	33·82	25·90	13·21	719·50	247·96
3342	—	—	—	—	—	—	—	—	—
3132	7·38	—	—	—	18·89	19·75	7·19	103·67	35·95
3022	1·81	2·47	3·01	2·45	2·68	2·87	2·30	2·56	3·08
2967	1·03	0·88	1·13	0·84	1·09	1·09	1·10	1·34	1·07
2893	1·00	1·00	1·00	1·00	1·00	1·00	1·00	1·00	1·00
2893	K = 7·50	12·77	20·60	4·16	6·30	3·90	22·40	1·64	4·84 min

undertaken, but in one experiment with *Staph. aureus* (Vollum) of two plates exposed on the same day with erythrosin 1/8000 and 1/16000 respectively, we found no effect in the line 5780 A in the latter concentration, and in the line 5461 A the same culture was 3·51 times more sensitive with the 1/8000 concentration.

We found in the microbes we tested that the gram-positive were more easily sensitized than the gram-negative microbes. Using the same lamp we found that, with the wave-lengths longer than 3132 A, there was no trace of effect in the sensitized plates in exposures of 32 minutes for *B. dys. Flexner V* (Mott), *V. Metchnikoff*, *B. dys. Flexner V* (37), and *B. dys. Flexner V* (45) and of 64 minutes for *B. coli* (Lister), while there was marked effect in *Staph. aureus* (Yellow) in 17 minutes, in *Staph. aureus* (White) in 12 minutes, in *Staph. aureus* (Vollum) in 8 minutes, in *C. Hofmannii* in 4 minutes, and in *Staph. albus* (Pasteur) in 2 minutes.

That not only the total, but also the relative, sensitivity of different lines was very different with the various microbes is shown in Table VII where in each experiment the time for 40% growth in each line was divided by the time calculated for the line 2893 A. The actual times for 40% growth in the line 2893 A are also given in Table VII. For *B. dys. Flexner V* (37) and (45), *Staph. aureus* (Yellow) and *Staph. aureus* (White) the longest exposures given were 190 minutes. The cultures of *B. dys. Flexner V* (37) and (45) were serologically the same and were both exposed on the same day, but it will be seen that in the line 5780 A *B. dys. Flexner V* (37) is 21·9 times more sensitive than *B. dys. Flexner V* (45); in 5461 A 8·2 times; in 3655 A 2·9 times; and in 3132 A 2·88 times, while in the line 3022 A it is slightly more resistant.

Staph. aureus (Yellow) and *Staph. aureus* (White) originally came from the same culture of a Staphylococcus aureus which, after long cultivation anaerobically, produced non-pigmented colonies, and it will be seen that, although the exposures were not made on the same day, there was no marked difference in their relative sensitivity.

Discussion of Results

Otto Rahn (1930, *a*) says that "the death of unicellular organisms is brought about by the inactivation of a certain number of essential molecules in the cell". It has been shown in our experiments that certain homogeneous species of microbes, viz., three strains of *Staphylococcus aureus* (Table III) and two strains of *S. Aertrycke* (fig. 5), have different curves. Two strains of *B. paratyphosus A*, serologically alike, have

different curves (fig. 6), and two strains of *B. dysenteriae Flexner V*, serologically the same although having identical curves, show a great difference in their relative sensitivity to the different long wave-lengths when sensitized with erythrosin (Table VII). The bactericidal power of the spectral lines is not proportional to their relative energies, and different wave-lengths show a selective absorption by the protoplasm of different cell structures. For sensitized microbes, allowing for the fact that those microbes which retain the gram stain are absolutely more sensitive to the long wave-lengths, it does not explain why their relative resistance in the different spectral lines should be so varied. Whether or not this is due to a different protein molecular structure in microbes, otherwise homogeneous, and which in some cases cannot be distinguished by serological tests, it is suggested that these differences may be detected by means of the reactions of the microbes when exposed to light.

It is not possible to make an actual comparison between the results of the colony counts of other workers and our own estimation with the photoelectric cell, but as we always read our plates after a 22-hours incubation, when the bacterial growth had reached a maximum, our experiments were always comparable with each other, and one microbe could be readily distinguished from another by the shape of its curves.

If the logarithms of the percentage of growth in our experiments were plotted against the actual times of exposure, it was found that the order of death was not exponential.

Staph. aureus (Vollum) and *Staph. aureus* (B. B.) had convex curves from 100 to 0 percentage growth, showing cumulative action. The curve for *Staph. aureus* (Eye 4) was convex from 100 to 10, and then showed a slight concavity from 10 to 0 percentage growth. The curve for *Staph. albus* (Pasteur) was concave from 100 to 35, convex from about 35 to 10 percentage growth, and then it became slightly concave again. On the other hand, the curves for *B. dys. Shiga* (Lister), *B. dys. Flexner W* (Logan), and *B. coli* (Lister) were all concave, showing a varying resistance to the light from 100 to 0 percentage growth.

The order of death by disinfectants, when the logarithm percentage growth was plotted against the reciprocal of the concentration of disinfectant, appeared to depend upon the type of disinfectant used. With lysol in bouillon the curves for *B. coli*, *Staph. aureus*, and *Staph. albus* were all convex to about 50% growth and then straight, while with formalin in bouillon the same microbes were concave over the whole range.

According to Rahn (1930, *b*), " the order of death is not a function of the killing agent but a property of the organism ", and, so far as the action of light was concerned, we found this to be true, but, as has been seen,

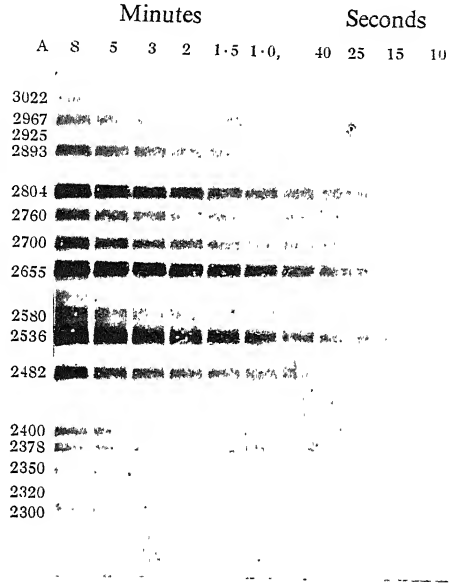

FIG. 1—*B. coli* (Lister). Spectral lines from 3132 to 2300 A. Times of exposure 10, 15, 25, and 40 seconds, 1, 1·5, 2, 3, 5, and 8 minutes.

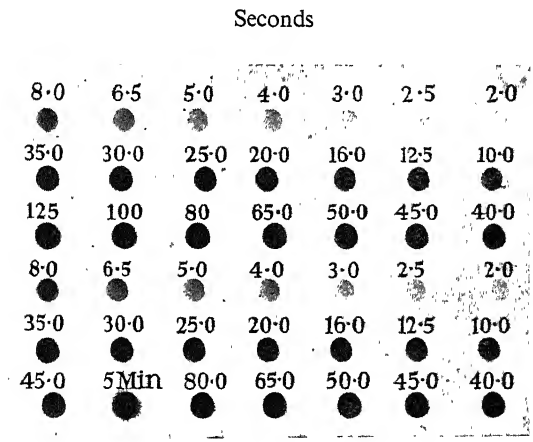

FIG. 2—*B. typhosus* (Hopkins). "Spot" 2655 A. Duplicate exposures. Times 2, 2·5, 3, 4, 5, 6·5, 8, 10, 12·5, 16, 20, 25, 30, 35, 40, 45, 50, 65, 80, 100, and 125 seconds. (In the second series the last exposures were 5 minutes, and 45 seconds, instead of the 100 and 125 second exposures.)

(*Facing p.* 470)

in the case of killing with disinfectants the shape of the growth curve was to some extent governed by the lethal agent employed.

I wish to express my thanks to the Medical Research Council for the funds which enabled us to make the " reading machine " and the " spectro-integrator " ; to Professor E. A. Milne, F.R.S., for the help he has kindly given me in dealing with the mathematics of this communication; to Dr. R. B. Bourdillon for his helpful criticism; and to Dr. R. L. Vollum for his great assistance in many ways.

Summary

Agar plates of different bacteria were exposed to a mercury vapour lamp and the percentage of growth after 22 hours' incubation calculated from readings made with a special apparatus. It was found that:—

With certain exceptions there was a difference in the shape of the curve for different bacteria.

The bactericidal effect in the spectral lines was not proportional to their relative energies.

Over a range of 1/1 to 1/8 the effect of reducing the intensity of light by screening showed that the time required for a given bactericidal effect was inversely proportional to the intensity of the light.

The relative bactericidal effect in the spectral lines appeared to vary according to the microbe exposed, but in all cases the line 2655 A was the most effective, followed in order by the lines 2536, 2804, 2482, and 2700 A.

The curves for some disinfectants on two species of microbes were determined, and a stimulation of growth in the higher dilutions was observed.

Experiments with bacteria sensitized with erythrosin and exposed to both the long and the short wave-lengths were made, and differences in effect in the different lines were observed. With the microbes used, it was found that the gram-positive organisms showed a much greater degree of sensitivity in the long wave-lengths than the gram-negative.

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Oxygen Dissociation Curves of Muscle Haemoglobin

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I—Introduction

The function of muscle haemoglobin in respect to its combination with oxygen has now been made clear. It has been shown independently by two observers, Theorell (1934) and the present writer (R. Hill, 1933), to have a higher affinity for oxygen than the haemoglobin of circulation. The muscle haemoglobin has the positions of maximum absorption in the visible region of the spectrum at 5815 A and 5446 A, while those of the haemoglobin of the blood are at about 5770 A and 5420 A. It can be easily shown that an extract of red muscle having its α band at about 5790 A (that is, in a position intermediate between the α bands of the two haemoglobins) gives on partial evacuation a shift of the α band to the red. This shows qualitatively the higher affinity for oxygen of the muscle haemoglobin. A convenient method of determining the dissociation equilibrium with oxygen on small amounts of dilute haemoglobin was then devised and applied to the problem. Meanwhile, Theorell achieved the isolation of muscle haemoglobin from the heart of the horse in a crystalline state and determined its dissociation curve by the established method for strong solutions. The results of these investigations are in agreement. Theorell's series of investigations on the muscle haemoglobin of the horse are as elegant as they are complete. The present paper describes experiments on the heart muscle haemoglobin of the ox, sheep, dog, and goose. The characteristic hyperbolic shape of the muscle haemoglobin dissociation curve being found in each case. The method of determining the dissociation curve is given in detail.

II—Method

A dilute oxyhaemoglobin solution saturated with atmospheric oxygen is added in successive amounts to haemoglobin of the same concentration contained in an evacuated vessel. The liquid is shaken to obtain gaseous

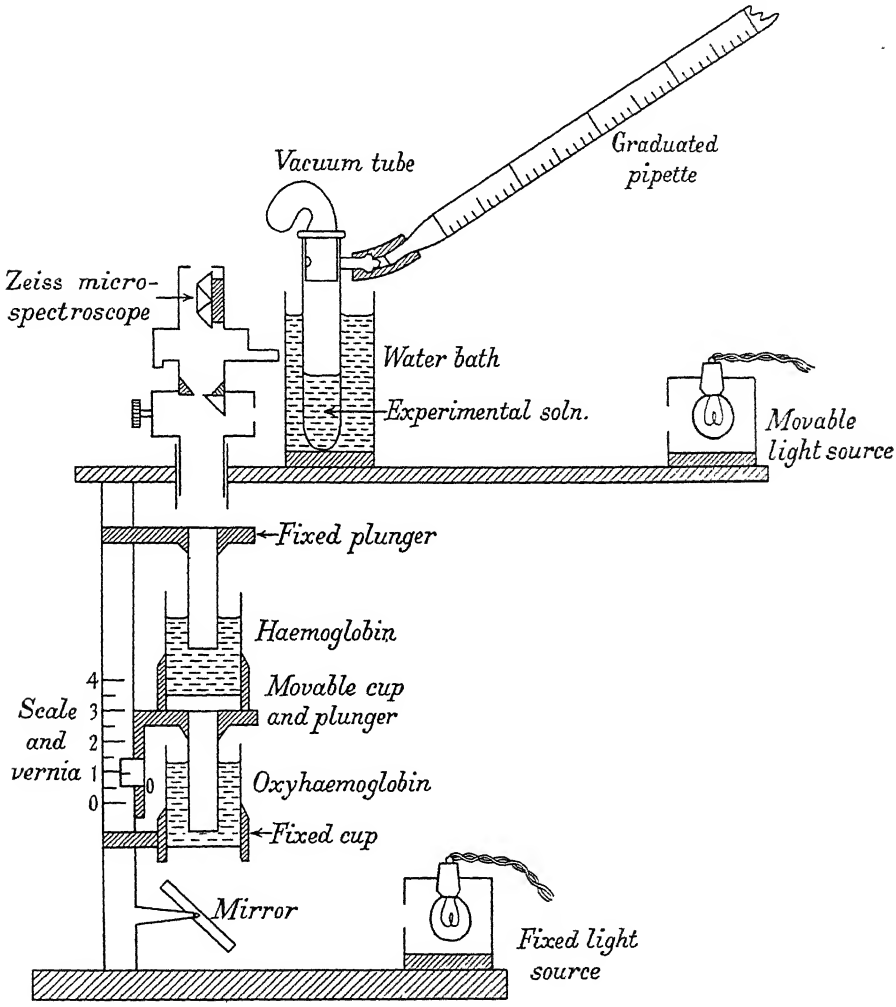

Fig. 1.

equilibrium, and spectroscopic determinations are made after each addition. The total pigment concentration in the tube is not altered during the experiment. We then have the required data for a dissociation curve.

The apparatus (fig. 1) consists of a Thunberg vacuum tube connected to a wide-mouthed, graduated pipette with a short length of pressure

tubing. Pressures of oxygen from zero to 20 mm of mercury can be obtained. For higher pressures of oxygen the tube may be simply exhausted to a measured pressure of air and water vapour and the oxygen pressure calculated. For spectroscopic estimation a Zeiss microspectroscope is used together with a single two-stage colorimeter attachment; being an extension of the apparatus described by Hill in 1929; the percentage of haemoglobin combined with oxygen in the vacuum tube is thus determined by visual comparison with optical mixtures of oxyhaemoglobin and haemoglobin having the same effective total concentration as the fluid in the vacuum tube.

The method is as follows. A stock solution of oxyhaemoglobin is made up corresponding to from $0 \cdot 8$–$0 \cdot 4 \times 10^{-4}$ gm atoms of Fe per litre in a suitable buffer. The solution is then shaken gently in a large vessel at room temperature and filtered through a folded paper in a funnel, avoiding splashing by allowing the fluid to run down the side of the receiving vessel. 5 cc of this fluid are then measured into a wet Thunberg vacuum tube, the stopper being secured with a little very stiff rubber grease. The completely oxygenated haemoglobin solution is then examined in the spectrum colorimeter arrangement, to make certain that the concentration is correct. The tube is then connected to a good water pump through a short length of wide glass tubing containing water to act as a trap. The tube is evacuated and tapped on the bench to cause the escape of bubbles of air. Before boiling and excessive frothing takes place, the tap is closed and the tube gently shaken for 2–3 minutes and then the tap is opened to the vacuum for an instant. The shaking is repeated, and when nearly all the air has been removed, there is little tendency to froth. The evacuation by this process is continued for 10 minutes, at the end of which time only solution and water vapour should be left in the tube, and very little water will have been removed by evaporation from the solution. It is necessary that the contents of the tube should be several degrees of temperature higher than the tap water running the suction pump, so that there is always a positive pressure of water vapour in the vacuum tube. After evacuation the pump is disconnected and the small amount of water contained in the glass connecting tube is sucked down the side tube of the vacuum tube, where it acts as a seal and ensures that no bubbles of air are entrapped in the grease on the exposed surface of the tap.

The completely reduced haemoglobin solution is then examined in the spectrum colorimeter arrangement, to make sure that its concentration is correct and that it is free from methaemoglobin, thus giving the zero point determination. A 22 mm length of pressure tubing is attached to the side

tube of the vacuum tube and filled by means of a drawn-out pipette with the oxygenated haemoglobin solution up to the top of the rubber, making sure that no air bubbles are included. A wide mouthed 10-cc graduated pipette filled with the oxygenated haemoglobin solution is fixed to the side tube by means of the rubber. Then on cautiously opening the tap a known amount of the haemoglobin solution is forced into the tube by atmospheric pressure. After shaking for 5 minutes, equilibrium is established, and the percentage of oxyhaemoglobin is estimated. With most solutions of haemoglobin, several additions can be made and the whole range of the dissociation covered in one experiment. At the end of the experiment air is admitted and the tube shaken in order to see if the original concentration of haemoglobin has been altered—that is, that the oxygen capacity has remained constant.

As a means of estimating the degree of saturation with oxygen of the haemoglobin, the principle of the spectrocolorimeter is used. The fluid to be estimated is compared against a variable optical mixture of oxy- and reduced haemoglobin of constant total pigment concentration, equivalent to the unknown. Usually a double wedge trough is employed for such a purpose, but the arrangement used on two-stage colorimeters is more convenient. This apparatus can easily be made from an old Dubosq colorimeter. The lower cup is fixed, and the lower plunger moves in the usual way, while a second cup is connected above the lower plunger and the second plunger is fixed above it. The distance between the lower cup and second plunger determines the total thickness of solution. The oxyhaemoglobin can be placed in the lower cup, and a solution of the same strength reduced with sodium hydrosulphite, in the upper cup: then on raising the plunger the percentage of oxyhaemoglobin can be varied from zero to 100%, and read off from the vernier scale. The nature of the absorption spectra makes the visual comparisons more accurate in the lower part of the scale. The spectroscope used is the Zeiss micro-spectroscope, use being made of the comparison prism. Two light sources are used (12 volt car head lights run from a transformer), the distance of one being variable. The vacuum tube containing the haemoglobin is placed in a small glass tank, containing water, opposite the comparison prism aperture.

The effective thickness of the fluid in the vacuum tube is previously determined by using oxyhaemoglobin and water in the two-stage colorimeter, and oxyhaemoglobin in the tube. As an example: the total thickness of haemoglobin solution in the colorimeter was 20 mm; while the vacuum tube used had an effective diameter of 13·8 mm; hence the fluid in the colorimeter must be $20 \div 13 \cdot 8 = 1 \cdot 45$ times as dilute as

the stock haemoglobin solution. The distance of the light source is adjusted so that the two spectra match both as regards intensity of bands and illumination of background. If any error has occurred in the equality of effective concentration this match is impossible at any position of the light source.

When the percentage of oxyhaemoglobin in equilibrium with the vapour phase has been determined the pressure of oxygen in the tube can be calculated. The total amount of oxygen introduced is known, and the oxygen will have divided itself between the water-vapour phase and the liquid. In the absence of haemoglobin the concentration of O_2 in the gaseous phase is about 30 times that in the liquid at room temperature. In the presence of haemoglobin it may be necessary to introduce a correction for the combined oxygen. It is most convenient to calculate the concentration of free oxygen in the liquid and convert it into pressure of oxygen by a factor.

The pressure of oxygen in the tube corresponding to the percentage of oxyhaemoglobin determined by the spectrocolorimeter can be calculated by the following formula:—

$$P \times \frac{v_1 [O_2] - (v_1 + v_2) [HbO_2]}{(v_1 + v_2) - A (v_0 - v_1 - v_2)}$$

where

v_1 = cc of fluid added from the pipette;

$[O_2]$ = total concentration of oxygen in the fluid added (*i.e.*, that dissolved plus that present as HbO_2);

$[HbO_2]$ = concentration of oxyhaemoglobin in the tube;

v_0 = volume of the tube;

v_2 = volume of fluid in the tube before evacuation;

A is the quotient of concentration of O_2 in gas divided by concentration of O_2 in liquid and is independent of pressure;

P is the factor for converting a concentration of dissolved oxygen into the equivalent pressure in mm Hg.

In the buffers used the solubility of oxygen was 94% of that in distilled water. For the determination of the solubility of oxygen in the buffer I am indebted to Professor H. Munro Fox. The constants A and P can be calculated from the available solubility data. Usually the concentration of haemoglobin used is low enough to be neglected. Then a graph can be plotted of v_1 and pressure of oxygen, which avoids the rather tedious calculation. Values of the constants used are shown in Table I.

For haemoglobin solutions sufficiently dilute, blood × 1/100, and with a not very high affinity for O_2, about 1 mm half saturated, there is no need to introduce the correction for the O_2 in combination with the haemoglobin.

TABLE I

Temperature °C	Molarity of dissolved oxygen at 760 mm pressure of air in buffer × 10^{-4}	A	P × 10^4
0	4·28	21·7	36·9
5	3·74	24·3	42·2
10	3·32	26·8	47·4
15	2·98	29·3	52·6
20	2·69	31·6	57·8
25	2·43	34·0	63·3
30	2·22	36·2	68·5
35	2·04	38·2	73·4
40	1·89	39·9	78·0

The pressure is then determined by

$$P \times \frac{v_1 [O_2]}{(v_1 + v_2) - A (v_0 - v_1 - v_2)}.$$

For each tube, values of the pressure of oxygen produced on adding a given volume of buffer solution saturated with air are calculated and a graph plotted from which the pressure values corresponding to cc of solution added can be read directly for each experiment.

The accuracy of the method is limited by the visual comparison of the two spectra. This may be within 3%, and should not deviate by more than 5%. The best concentration of haemoglobin is, for a tube of 14 mm effective thickness, normal human blood diluted 125 times, which is equivalent to $0 \cdot 8 \times 10^{-4}$ gm atoms of iron per litre.

The advantages of the method are the comparative rapidity of obtaining several points on the dissociation curve, and being able to use very low pressures of oxygen. The apparatus can be assembled in a portable form, and is not unduly bulky. The method is applicable to dilute suspensions of corpuscles and can be modified for a concentration equivalent to human blood diluted five times.

PREPARATION OF THE HAEMOGLOBIN SOLUTIONS

The heat was perfused with warm Ringer through the aorta. In the case of the ox, 2 canulae were introduced into the coronary arteries. The

heart after perfusion was dried on a cloth, freed from fat and ligaments, and minced. It was then extracted with an equal volume of 0·9% sodium chloride for ½-hour at room temperature and the fluid obtained by centrifuging filtered through kieselguhr. This fluid was then adjusted to approximately the p_H required and mixed with an equal volume of buffer.

This simple extract of muscle gave a dissociation curve very nearly the same as a more purified solution and as the crystalline haemoglobin of the horse heart obtained by Theorell. This is to be taken as evidence that the haemoglobin is not altered by further purification after it is extracted from the muscle. Results are given in Table II.

TABLE II—CRUDE EXTRACT OF PERFUSED OX HEART, p_H 9·2, 21° C; BAROMETER 760 mm; VOLUME OF VACUUM TUBE 23·9 cc; CONCENTRATION OF Hb = 10^4 GM ATOMS OF FE PER LITRE

Pipette added cc	Colorimeter scale reading		% HbO_2	mm O_2
0·25	0·15	0·13	7	0·05
0·50	0·31	0·31	15	0·10
1·00	0·54	0·57	28	0·20
2·00	0·70	0·70	35	0·47
4·00	1·19	1·13	58	1·15
9·00	1·43	1·35	90	3·70

The simple extract of muscle at p_H 7 usually had a slight oxygen uptake, which made observations on the dissociation curve impossible. Also it was found to contain small amounts of the haemoglobin of circulation. The haemoglobin was extracted as described previously, neutralized to p_H 7, and the bulk of the other proteins removed by the addition of basic lead acetate as in Theorell's preparation. Most of the remaining haemoglobin of the blood was removed and the solution no longer had an oxygen uptake. If the procedure was rapid a negligible amount of methaemoglobin was formed.

Muscle haemoglobin was prepared by the method described and compared with dilute solutions of the haemoglobin of circulation. Fig. 2 shows that the effect of change in hydrogen ion concentration is much less than in the case of the blood pigment. In fig. 3 the muscle haemoglobin of the heart of sheep and goose are compared. The muscle haemoglobin of the goose, like the haemoglobin of its blood, has less affinity for oxygen than that of the sheep. In fig. 4 the logarithms of the pressures of oxygen are plotted against the logarithms of the ratio of

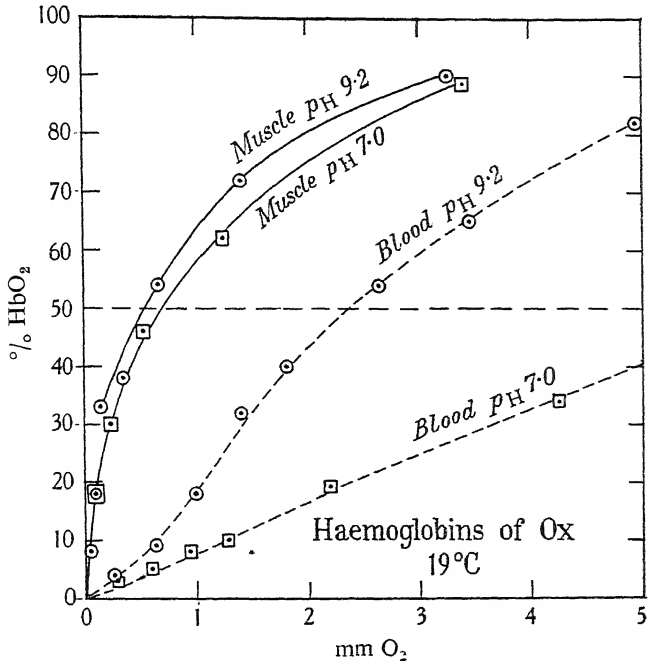
100
90
80
70
60
50
40
30
20
10
0
% HbO2
Muscle pH 9·2
Muscle pH 7·0
Blood pH 9·2
Blood pH 7·0
Haemoglobins of Ox
19°C
0
1
2
3
4
5
mm O2

FIG. 2.

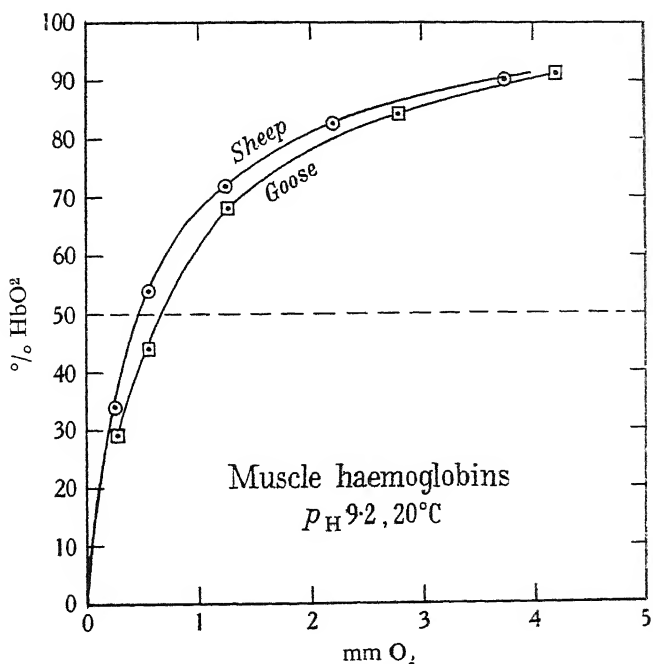
100
90
80
70
60
50
40
30
20
10
0
% HbO2
Sheep
Goose
Muscle haemoglobins
pH 9·2, 20°C
0
1
2
3
4
5
mm O2

FIG. 3.

HbO_2 to Hb. The muscle haemoglobins of the dog and ox give approximately straight lines of slope 45° corresponding to a hyperbolic curve. The blood haemoglobins give a curve corresponding with the S-shaped

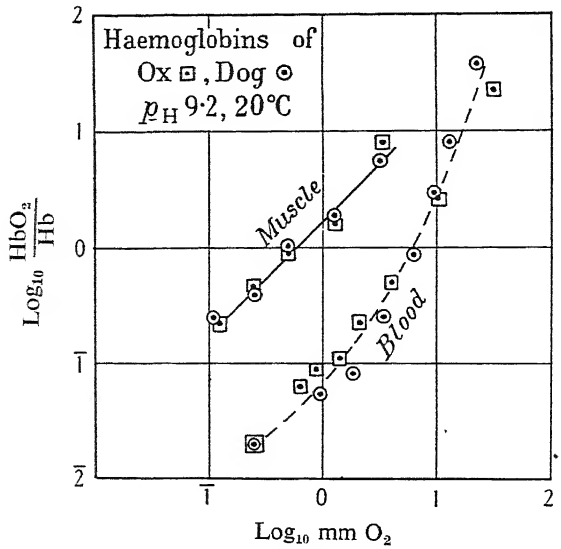

FIG. 4.

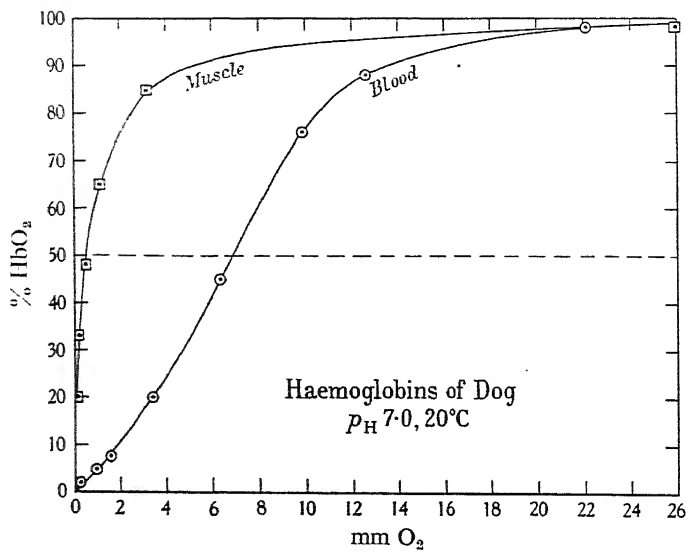

FIG. 5.

form. In fig. 5 the muscle and blood haemoglobins of the dog are compared in dilute solution at p_H 7. In each case it is shown that at lower oxygen pressures the muscle haemoglobin has a much higher affinity for

oxygen than the haemoglobin of the blood. At higher oxygen pressures it is seen that the curves cross—a consequence of the different nature of the shape of the curve.

III—Discussion

Keilin (1925) has shown that the distribution and relative concentration of cytochrome in muscles corresponds with the respiratory activity, and also that cytochrome plays the part of a catalyst in the oxidation process of respiration. The cytochrome is oxidized by the indophenol oxidase which reacts directly with molecular oxygen. The respiratory pigment haemoglobin supplies molecular oxygen directly to the tissues by diffusion. Whenever muscle haemoglobin is found in a muscle there is always a relatively high concentration of cytochrome, and the muscle is capable of a high rate of respiration. On the one hand, the work of Warburg (1929) on the respiration of yeast and Keilin's experiments on cytochrome and the indophenol oxidase (1925, 1930) of muscle show that the oxidase can function with a very low pressure of oxygen—about 5 mm of mercury. On the other hand, the pressure of oxygen in the venous blood of man is 40 mm of mercury. The dissociation curve of muscle haemoglobin lies between these two limits of pressure. At the oxygen pressure of venous blood it is 94% saturated with oxygen (*see* fig. 6), at 5 mm it is 60% saturated. The diagram, fig. 6, is an extension of Barcroft's figure (1928).

The presence of muscle haemoglobin must be regarded as a means of storing oxygen. The supply of oxygen to a muscle may be intermittent, the capillaries being compressed during a contraction. Reference to fig. 6 will show that the oxygen available is 28% of the amount in combination with the muscle haemoglobin. In a mammalian heart containing 0·5% of muscle haemoglobin (Watson, 1935) 2·0 cmm of oxygen from the muscular haemoglobin would be evolved per gram of muscle during a fall of pressure of oxygen from 40 to 5 mm Hg.

The fluid contained in the muscle would yield 0·9 cmm of oxygen from physical solution over the same fall of pressure. The total reserve of O_2 would then be 2·9 cmm. The oxygen consumption of heart may be as much as 9700 cmm per hour per gram (Evans and Matsuoka, 1915), and if the beat is 1 second, 2·7 cmm of O_2 per gram would be required for each recovery.

If the curve of muscle haemoglobin represents that in the living muscle, the storage of available oxygen is determined by the position of the dis-

sociation curve and the quantity of muscle haemoglobin present.* Diffusion may be supplemented by the movements due to the contracting muscle.

If the heart beat were more rapid—*e.g.*, five times—while the oxygen uptake remained the same, then there might be enough oxygen in solution in the tissue itself to act as a reserve. In small mammals with rapid heart beat muscle haemoglobin is often absent. Here, as Keilin (1925) has found, cytochrome is present in great amount; and here the catalytic activity is more essential than the storage. An extreme case is that of the wing muscles of insects which have the largest amount of cytochrome of

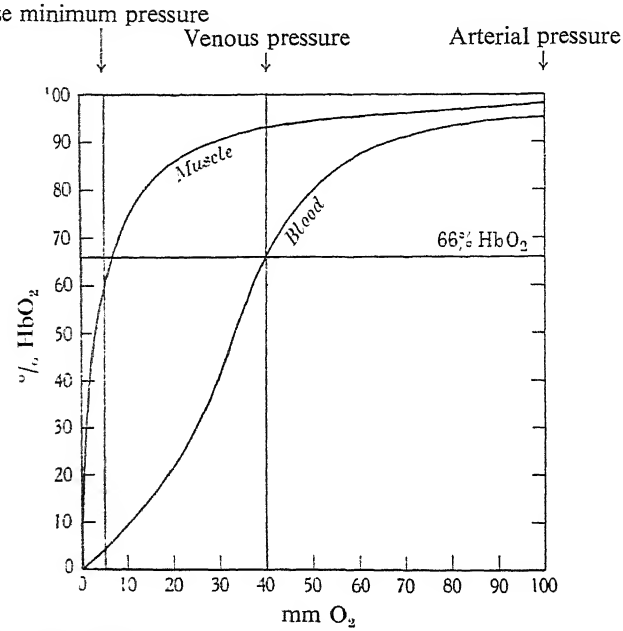

FIG. 6.—The relationships of the dissociation curves of muscle haemoglobin and of blood to the minimum oxygen pressure required by the tissue.

any tissue but do not contain muscle haemoglobin. The radula muscle of *Helix pomatia* contains much cytochrome but not muscle haemoglobin; certain marine gasteropods, however, have both muscle haemoglobin and cytochrome in the jaw muscles.

The presence of muscle haemoglobin can be regarded as an indication of the magnitude of the oxygen utilization in a rapid recovery period of the muscle, the amount of cytochrome being an indication of the Q_{O_2}. In

* [*Note added in proof, June* 16, 1936.—Since going to press, complete data of the kinetics of the muscle haemoglobin of the horse have been published by Millikan (1936). He points out that the rapidity of reaction with oxygen is amply sufficient for available oxygen storage between the beats of a mammalian heart.]

the process of respiration the muscle haemoglobin acts as a carrier of molecular oxygen between the haemoglobin of circulation and the indophenol oxidase cytochrome system in the cell.

IV—Summary

A spectroscopic method of determining the dissociation curve of small amounts of haemoglobin is described.

A means of obtaining accurately very low pressures of oxygen is described.

The haemoglobins of the heart muscle of ox, sheep, dog, and goose give similar hyperbolic curves little influenced by changes in p_H and having a much higher affinity for oxygen than the haemoglobins of the blood.

The physiological function of muscle haemoglobin is discussed in relation to the oxygen supply of cellular respiration. It is suggested that the muscle haemoglobin indicates a presence of a reserve of oxygen not only in cases of intermittent supply but also in cases of intermittent consumption of oxygen.

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The Oxygen Dissociation Curve of Haemoglobin in Dilute Solution

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(*Communicated by Sir Frederick Hopkins, O.M., F.R.S.—Received April* 8, 1936)

I—INTRODUCTION

The spectroscopic determination of the oxygen dissociation curves of haemoglobin has an advantage over the tonometer and gas analysis method, in that much smaller quantities of haemoglobin can be made use of. The spectroscopic method was used to determine the relationship between the foetal and maternal haemoglobins in the sheep during a study of foetal respiration made by Barcroft (1935). The conditions for the comparison of the haemoglobins were a dilute solution of the haemoglobin at p_H 9·2 (borate buffer) and at 20° C. These conditions were chosen because of the very accurate determinations of the dissociation curves of dilute haemoglobin of the sheep by Forbes and Roughton (1931) and because these authors recommend p_H 9·2 at room temperature as most suitable for a study of the oxygen equilibrium of haemoglobin, all the haemoglobin being in the form of the alkali salt. McCarthy (1933) and Hall (1934) had found previously that the haemoglobins of the foetal and maternal goat were different, the foetal haemoglobin (in the blood and as purified haemoglobin) having a higher affinity for oxygen. The same relationship was found to exist in the sheep haemoglobins in dilute solution at 20° C and p_H 9·2. When samples of human foetal and maternal blood (sent by Professor Fleming from the Obstetrical Department of the Royal Free Hospital) were compared in dilute solution it was found that the foetal haemoglobin had a lower affinity for oxygen than the maternal. This was also found by Haurowitz (1935) for dilute solutions of the haemoglobins of mother and new born infant. Haurowitz, however, pointed out that in the corpuscles the affinity for oxygen is less in the infant's haemoglobin than in that of the mother, but the method used by him did not allow of measurements on suspensions of corpuscles. In the present work the dissociation curves of dilute suspensions of corpuscles have been compared with similar solutions of the haemoglobin. It was found that the relationship of the dissociation curves for

human foetal and maternal corpuscles is the same as that found by Barcroft in the goat and in the sheep. It has now been found that by a dilution of human adult haemoglobin the dissociation curve is altered by 200% to a position of higher affinity for oxygen, without any marked change in shape. The haemoglobin of the human foetus, on the other hand, is much less affected by dilution, thus explaining the anomaly of the reversed relationship when solutions of the haemoglobins are used instead of suspensions of corpuscles.

It was shown by the work of Bock, Field, and Adair (1924), and by Adair (1925), that a solution of haemoglobin free from stromata and of a similar concentration to blood gives a dissociation curve like whole blood. This makes it clear that in the comparison of dilute haemoglobin solutions with suspensions of corpuscles we are concerned, not simply with a change in the haemoglobin due to haemolysis, but a change due to a dilution of the contents of the corpuscle.

The difference between blood or corpuscles and a solution of haemoglobin was observed by Forbes and Roughton (1931) in the sheep, but they suggested that the difference might be due to a difference in the p_H of the corpuscles, which were used in the whole blood.

The present paper deals with experiments on the haemoglobin in solution and in the corpuscles in certain animals.

II—Methods

The dilute solutions of haemoglobin were prepared by adding the whole blood to a large volume of distilled water (0·5 cc of blood to 50 cc of water) and after half an hour adding an equal volume of N/5 buffer. The dilute suspensions of corpuscles were made by adding the whole blood to 1% sodium chloride and adding an equal volume of N/5 buffer. The more concentrated solutions were prepared either by laking whole blood with saponin (which was found to have no influence on the dissociation curve) or in one case by laking the corpuscles with ether and preparing the haemoglobin by Adair's method (1925).

The dissociation curves of the dilute solutions and suspensions of corpuscles were determined by the spectroscopic method in Thunberg tubes 1·6 cm in diameter. The oxygen pressures were obtained either by a mercury manometer, having a syphon at the base to allow reading the lower meniscus, or by the method of the addition of oxygenated solution described in the previous paper. These two methods were tested out on the same sample of haemoglobin and were found to give the same result. The dissociation curves of the more concentrated

solutions were determined in a specially designed vessel. The vessel (fig. 1), which is made of two pieces of glass tubing, consists of a larger portion at the top with a rubber bung and tap, and a smaller portion at the bottom into which a sealed tube containing water closely fits. The small amount of haemoglobin solution is brought into equilibrium with a known pressure of oxygen by rotating the vessel for 10–15 minutes. Then the sealed tube is allowed to slide in to the lower vessel, thus producing a thin film of the haemoglobin. Spectroscopic readings are then made in the same way as in the case of the Thunberg tube, only the vessel is turned about the vertical axis and readings taken in several positions to compensate for any irregularities in the bores of the closely fitting glass tubes. The minimum thickness found convenient was 0·4 mm. With a smaller difference in the internal and external diameters of the tubes, the apparatus is difficult to manipulate.

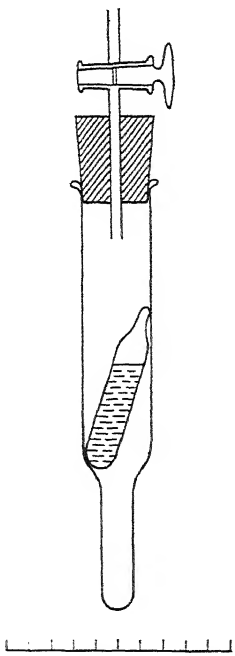

FIG. 1.

The buffers were used in relatively high concentration N/10 to avoid changes in p_H. In the case of dilute solutions and suspensions the p_H was determined electrometrically after the experiment.

III—Comparison of a Dilute Suspension of Human Corpuscles in Buffer with Whole Blood

The blood (H. P. W.) was drawn from a vein and treated with neutral potassium oxalate. The dissociation curve was determined in the usual way with the van Slyke method at 40 mm pressure of CO_2 and at 37° C. The new tonometers after Barcroft (1934) were used. The corpuscles were suspended at a dilution of blood 1 100 in 1% sodium chloride and an equal volume of phosphate buffer p_H 7·46 was added. The dissociation curve of the dilute suspension was determined by the spectroscopic method at 37° C. The result is shown in fig. 2.

IV—The Effect of Keeping a Dilute Solution of Human Haemoglobin

The blood (R. H.) was allowed to drop directly into distilled water and diluted a hundred times. Before each experiment an equal volume of M/5 phosphate buffer p_H 7·4 was added to a portion. During an

interval of 4 hours at room temperature there was no change in the position of the curve. The half-saturation point was 3·8 mm at p_H 7·4 and 23° C. On keeping the solution 4 days in the dark at 0° C the half-saturation point was 2·0 mm at p_H 7·4 and 23° C. After another three days the curve showed no further change. It was found, during the course of many experiments on the fresh dilute haemoglobin of the same subject, that the curve occasionally had a position of higher affinity towards the position of the curve produced on keeping the solution. These irregularities, which at present cannot be explained, were only found in phosphate at p_H 7·4, and not in borate at p_H 9·2.

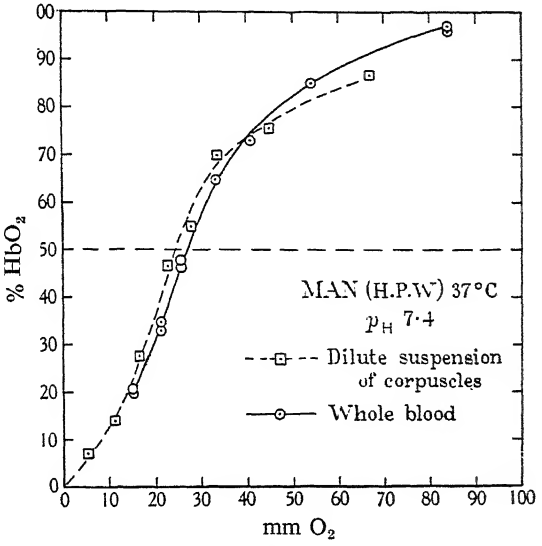

FIG. 2.

V—Comparison of a Dilute Suspension of Corpuscles and a Solution of Haemoglobin in Various Animals

The measurements were made at a 200-fold dilution of blood at p_H 7·4 and 9·2. It is seen from the curves (fig. 3) that the effect of dilution is greatest in the rat, guinea-pig, and man, while in the cat there was very little effect. Dilution at p_H 9·2 produces a similar effect to dilution at p_H 7·4.

In the case of the hen (fig. 4) it is seen that the effect of dilution is in the same direction as in the mammals. Christensen and Dill (1935) found that a concentrated solution of the haemoglobin had a slightly less affinity for oxygen than the whole blood at the same temperature, CO_2 pressure, and similar alkali reserve.

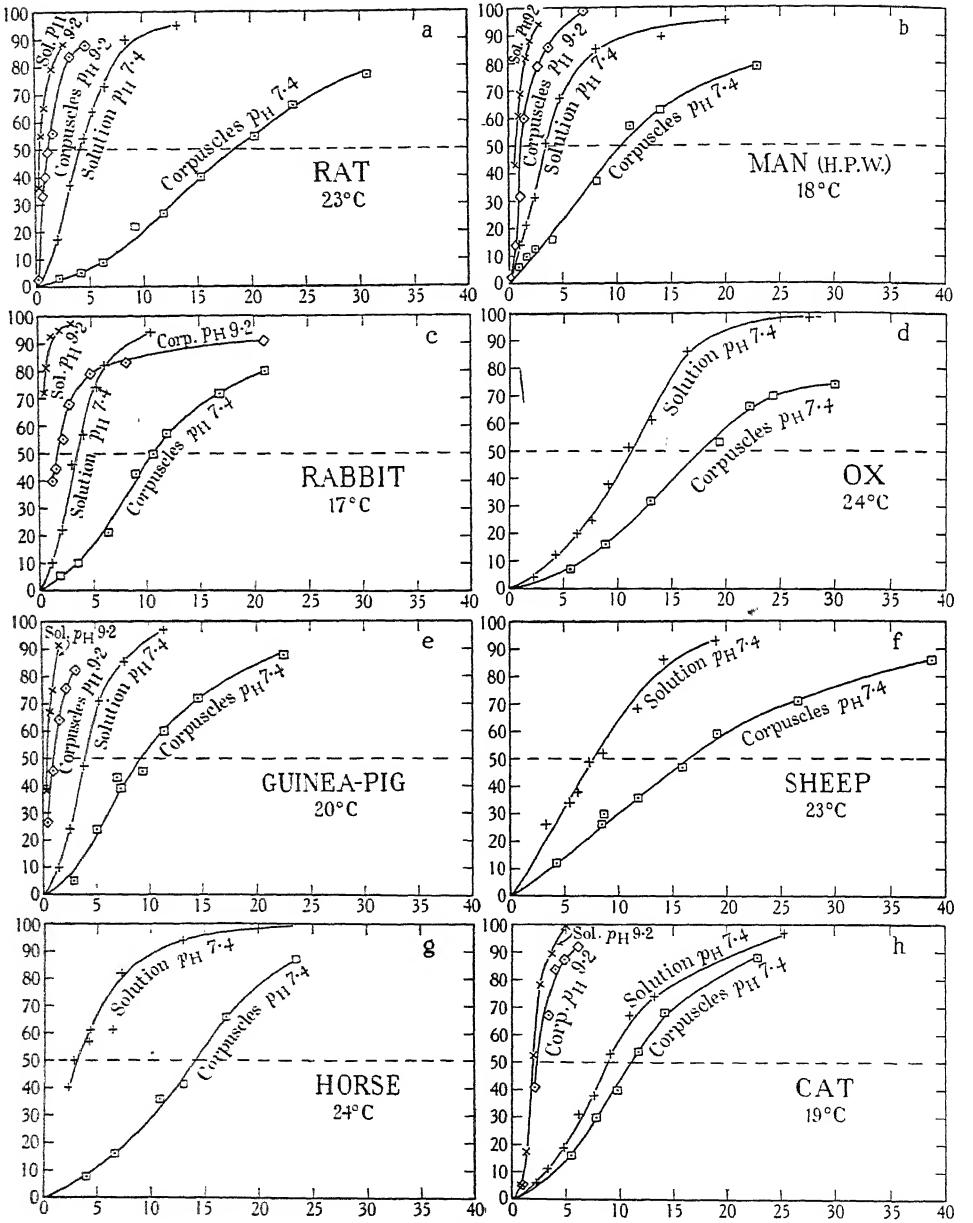
a
Sol. pH 9·2
Corpuscles pH 9·2
Solution pH 7·4
Corpuscles pH 7·4
RAT
23°C
b
Sol. pH 9·2
Corpuscles pH 9·2
Solution pH 7·4
Corpuscles pH 7·4
MAN (H.P.W.)
18°C
c
Sol. pH 9·2
Corp. pH 9·2
Solution pH 7·4
Corpuscles pH 7·4
RABBIT
17°C
d
Solution pH 7·4
Corpuscles pH 7·4
OX
24°C
e
Sol. pH 9·2
Corpuscles pH 9·2
Solution pH 7·4
Corpuscles pH 7·4
GUINEA-PIG
20°C
f
Solution pH 7·4
Corpuscles pH 7·4
SHEEP
23°C
g
Solution pH 7·4
Corpuscles pH 7·4
HORSE
24°C
h
Sol. pH 9·2
Corp. pH 9·2
Solution pH 7·4
Corpuscles pH 7·4
CAT
19°C

FIG. 3.

VI—Effect of Temperature on the Haemoglobin of Man (R. H.) in a Suspension of Corpuscles and in a Dilute Solution

The experiments were carried out in phosphate buffer at p_H 7·3. In the diagram, fig. 5, the $\log_{10}$ oxygen pressure in millimetres Hg for 50%

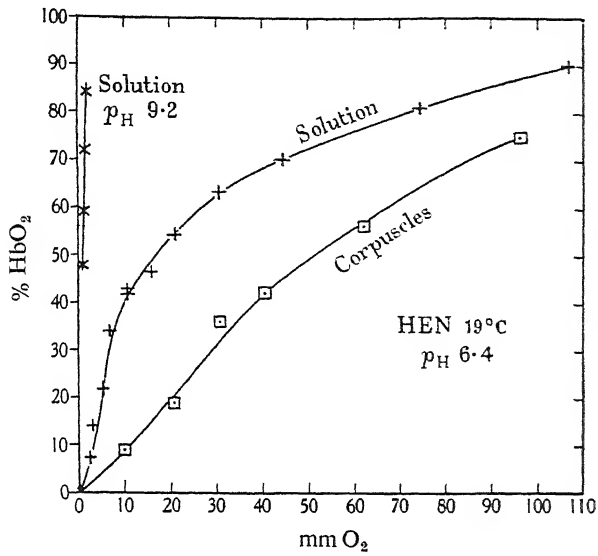

Fig. 4.

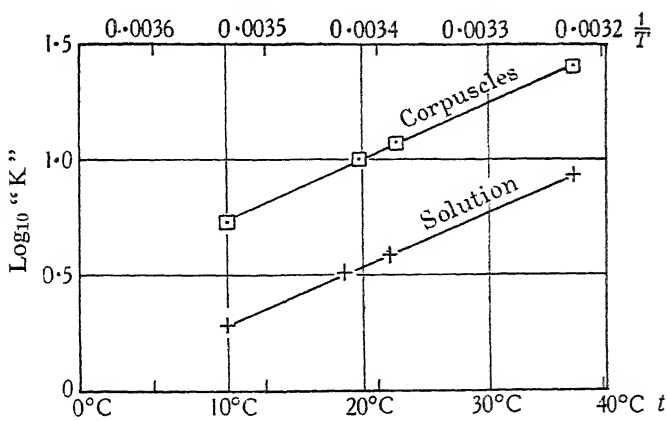

Fig. 5.

saturation is plotted against the reciprocal of the absolute temperature. The effect of alteration in temperature is nearly the same for the corpuscle suspension and the dilute solution (blood × 1/200).

TABLE I

Solution			Corpuscles		
°C	mm Hg O_2	% saturation	°C	mm Hg O_2	% saturation
10	0·8	20	10	1·0	8
	1·4	37		1·8	19
	2·1	69		5·6	48
	3·5	84		8·0	74
18·5	1·0	6	19·5	0·9	2
	1·6	16		2·5	8
	2·5	35		3·5	13
	3·5	54		4·9	20
	4·9	72		6·8	30
	8·1	93		9·6	47
	14·2	95		14·2	72
				22·9	91
22	2·2	35			
	3·6	54	22·5	3·8	12
				7·6	28
37	2·3	5		11·8	54
	3·8	19		15·3	69
	7·1	39			
	8·3	48	37	8·0	7
	9·6	53		8·8	8
				12·4	16
				16·0	30
				27·1	54
				69·8	94

VII—THE EFFECT OF CERTAIN SUBSTANCES ON THE DISSOCIATION CURVE OF DILUTE HUMAN HAEMOGLOBIN

(*a*) *Corpuscle Dialysate*—Horse corpuscles, washed with sodium chloride solution, were laked with saponin, and in another case by freezing. They were dialysed in cellophane against an equal volume of distilled water for 48 hours. The dialysate, when added to a dilute solution of human haemoglobin in an equal amount, produced a shift in the curve of the dilute solution at p_H 7·4 towards that of the stronger solution by about 25% of their difference (fig. 6). At p_H 9·2 the dialysate produced no change on the position of the curve. If the corpuscles were simply dialysed against an equal volume of distilled water (when complete haemolysis did not occur), the dialysate (though containing SH compounds) did not affect the dissociation curve of the dilute haemoglobin.

The dialysate could be kept at 0° C with chloroform for four days without losing its activity but could not be kept indefinitely. It produced an effect after the nitro prusside reaction for — SH had become negative. Boiling was found to inactivate it, and its property could not be restored by leaving in contact with CO_2 (60 mm pressure). Dilute sodium bicarbonate added to the haemoglobin solution produced no effect on the dissociation curve if the p_H was unaltered. The dialysate also contained the — SH compounds of the red corpuscle. Pure GSH in a concentration of 0·03% and 0·3%, however, produced no shift in the curve of dilute haemoglobin, but it did appear to alter the shape to a slightly more S form

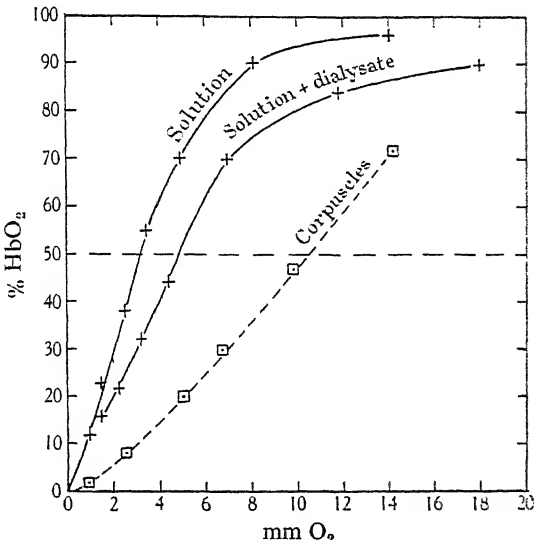

FIG. 6.

(fig. 7). It was thought that the effect of the dialysate on the dissociation curve might be due to reducing substances. If during an experiment there is an oxygen uptake, owing to the method, the dissociation curve will appear to represent a lower affinity for oxygen. By keeping the mixture, however, at equilibrium over a relatively long period, a slow oxygen uptake can be detected. A rapid oxygen uptake will give irregular results. No evidence of oxygen uptake could be found in the presence of the dialysate or with glutathione under the conditions of the experiments. As was stated earlier, the dilute haemoglobin of man may change further to a position of still slightly higher affinity. The dialysate from horse corpuscles affected this in the same proportion as it affected the normal curve.

(*b*) *Potassium and Sodium Ions*—As the relative amounts of K and Na vary in the corpuscles of different species (Abderhalden, 1923), it was necessary to find out if the position of the curve of the dilute haemoglobin was influenced by the presence of either one or other of these cations (*cf.* Barcroft and Camis, 1909).

Fig. 7 shows the effect obtained on dilute human haemoglobin in phosphate buffer, with sodium phosphates only and with potassium phosphates only. There was both a small shift and a change in shape.

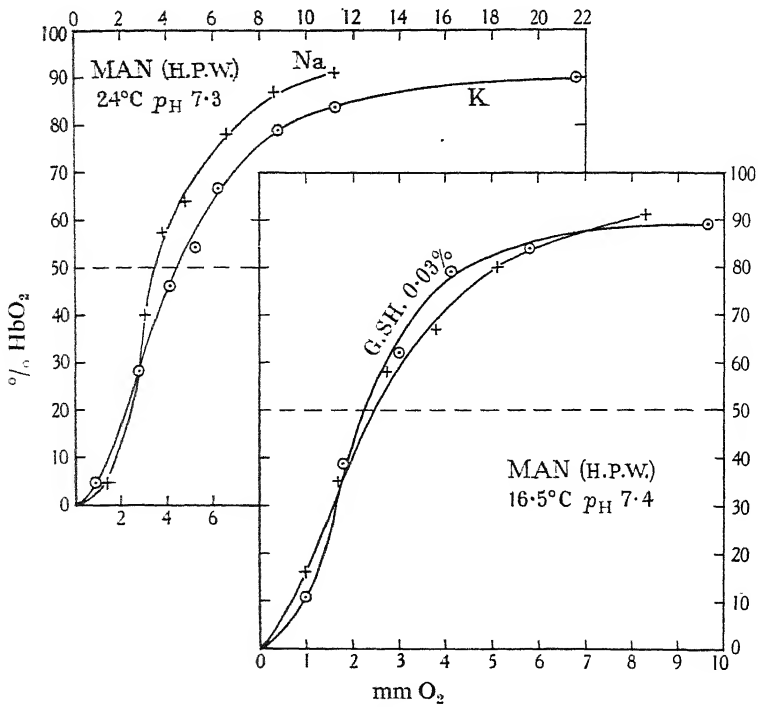

FIG. 7.

VIII—THE EFFECT OF CONCENTRATION ON THE DISSOCIATION EQUILIBRIUM OF OXYHAEMOGLOBIN

Human haemoglobin was found to be affected at a greater concentration than the haemoglobin of the sheep. Some results are given in Table II. While the sheep haemoglobin may be diluted to a concentration of 1/50th of that in the whole blood without much change, human haemoglobin was found to have the affinity for oxygen completely changed at a concentration of 1/5th of that in the whole blood.

TABLE II

Species	Temperature °C	p_H	Dilution relative to blood	mm Hg pressure of O_2 for half-saturation
Man	19	7·4	Corpuscles	9·8
,,	19	7·4	200	3·2
,,	19	7·4	5	3·1
Sheep (*a*)	23	7·4	100	9
,,	23	7·4	50	19
,,	23	7·4	4	24
Sheep (*b*)	23	7·2	100	8
,,	23	7·2	Corpuscles	17

IX—DISCUSSION

The results described in the present paper must be considered in relation to our definition of the specificity of haemoglobin with its affinity for oxygen. It would have been perhaps desirable to refer in each case to the properties of a dilute solution of haemoglobin. It is clear, however, that at present we must use either the corpuscle or a strong haemoglobin solution as a standard of reference. In the animals examined here it can be seen that the range of variation caused by diluting the haemoglobin of one animal may be greater than the variations between the corpuscles of a series of different animals. Moreover, there seems to be no simple relation between the absolute position of the dissociation curve of the corpuscles and the relative shift produced by a great dilution of the haemoglobin.

The increase in affinity for oxygen, when haemoglobin is diluted, is not accompanied by any marked change in the shape of the curve, which preserves its S-shaped character up to a dilution equivalent to blood diluted 460 times. The degree of inflexion is conveniently measured by the slope of the curve obtained by plotting the logarithms of the oxygen pressures with the logarithms of the ratio of the HbO_2 to the Hb in equilibrium. Adair (1925) has shown that the slope is not constant for strong solutions of haemoglobin, and this was also found by Forbes and Roughton (1931) for the dilute haemoglobin of the sheep. In fig. 8 the curves are shown for a suspension of human corpuscles at p_H 7·3 at 19·5° C and a solution of haemoglobin (blood × 1/200) at p_H 7·3 and 18·5° C (*see* Table I). It is seen that the slopes of the curves are similar. The curves for $n = 1$ and $n = 2$ are given for reference, n being the " average order of reaction " coefficient in the equation of A. V. Hill.

The work of Barcroft on foetal respiration and that of R. Hill (1933) and Theorell (1934) on muscle haemoglobin has shown a definite adaptation of the position of the dissociation curve of haemoglobin to the physiological conditions of oxygen transport. Both foetal haemoglobin and muscle haemoglobin have a higher affinity for oxygen than the haemoglobin of circulation. By a relatively small dilution—five times—of the haemoglobin of human blood a very large increase in affinity is obtained outside the organism.

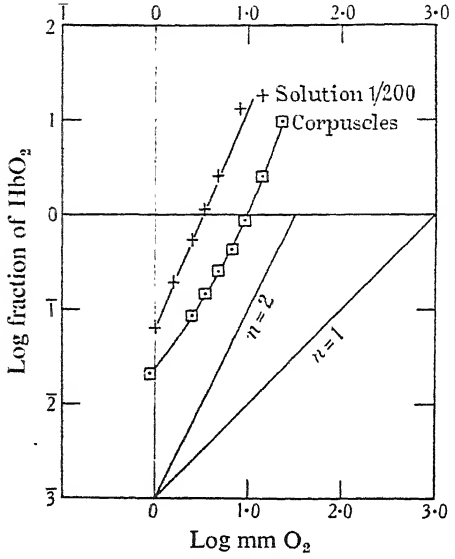

FIG. 8.

We wish to record our thanks to Sir Frederick Hopkins and to Sir Joseph Barcroft for their interest in this work and for the facilities they afforded us. We are very grateful to Dr. G. S. Adair for helpful suggestions. One of us (H. P. W.) is indebted to the "Bachiene-Stichting" Leiden, Holland, for a grant. We are grateful to Dr. N. W. Pirie for a sample of pure glutathione.

X—SUMMARY

Corpuscles of human blood in a dilute suspension in buffer at p_H 7·4 and 37° C give nearly the same dissociation curve as blood with 40 mm CO_2 pressure at 37° C.

Human haemoglobin in buffer at p_H 7·4 and at a dilution of from 5 to 200 times normal blood has a threefold increased affinity for oxygen.

The diluted haemoglobins of different animals show widely different

variations from the respective corpuscles, but the change on dilution is always towards a higher affinity.

In man, the temperature coefficient is nearly the same for the corpuscles and the dilute solution.

The dialysate, from laked corpuscles of the horse, influences the dissociation curve of dilute human haemoglobin, moving it towards the curve of the corpuscles.

The change in the position of the dissociation curve takes place at greater concentrations of haemoglobin in the case of man than in the case of the sheep.

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578.085.23:611.018.4

The Growth of Embryo Bones Transplanted Whole in the Rat's Brain

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(*Communicated by Sir Arthur Keith, F.R.S.—Received* 14 *March*, 1936)

[Plate 14]

An earlier study (Willis, 1935) showed that in rats the brain is a very favourable nidus for the growth of implanted embryo tissues. Following the introduction of small quantities of embryo mince, large masses of bone and cartilage, epidermal, and mucosal cysts, salivary and other glands, skeletal muscle, and teeth were obtained; and in the most successful implants the rate of growth and differentiation approximated to that of an intact animal.

The perfect growth of complete teeth in these experiments suggested that it might also prove possible to grow complete bones in the same way. This hope has been realized; it has proved possible, following the intracerebral implantation of early cartilaginous primordia of the limb buds of young embryos, to obtain well-formed long bones such as femur or tibia nearly 2 cm long in the brains of the host rats. In some of the experiments two long bones with an intervening joint have developed.

The technique was generally similar to that described in the previous paper. From fresh embryos, kept moist by normal saline, the desired cartilaginous parts were carefully dissected out with a minimum of injury, freed as far as possible from surrounding soft tissues, loaded into the end of a lumbar puncture needle, and thrust into the right cerebral hemispheres of the host rats. These were killed 8 to 10 weeks later, their brains were removed and fixed in formalin solution; skiagrams of the brains were obtained, and they were then dissected to expose and free any bones within them. Longitudinal microscopical sections of some of the best formed bones were prepared.

Experimental Results

Details of some preliminary experiments are omitted. In these, various skeletal parts of 15 mm embryos were implanted into the brains of six

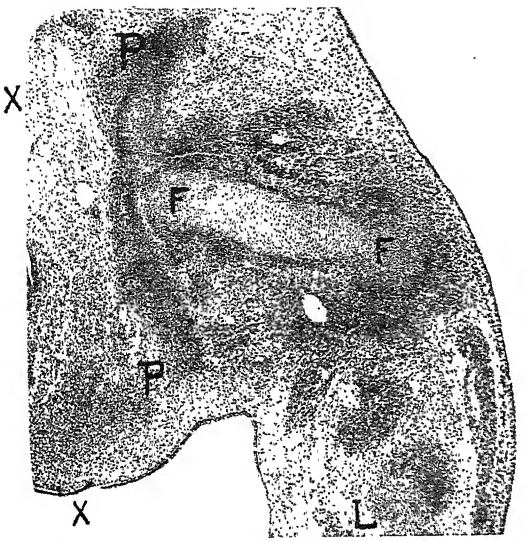

FIG. 1.

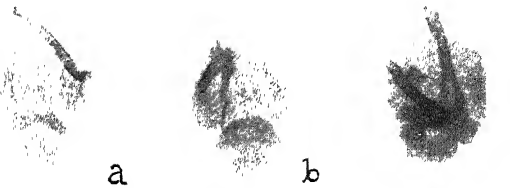

FIG. 2.

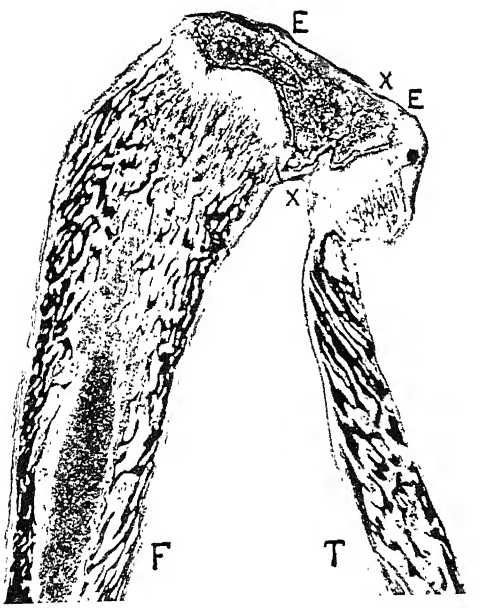

FIG. 4.

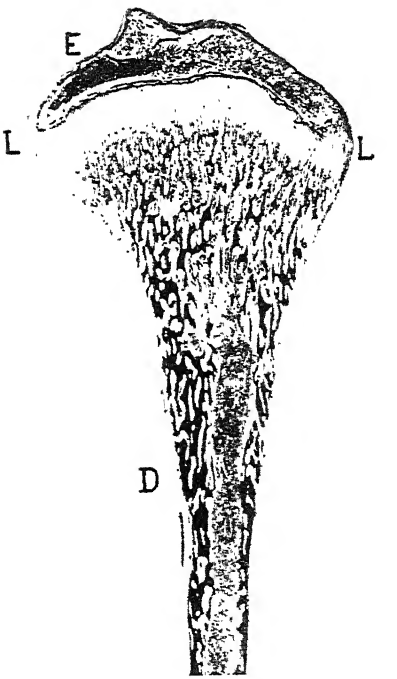

FIG. 3.

weeks old hosts or of adult rats. Although masses of bone grew in many of the hosts' brains, their shapes were usually irregular and unlike that of the normal bones. When fully grown adult rats were used as hosts, frequently no bony masses at all developed from the implants.

The most successful experiment was as follows. The implants were the cartilaginous rudiments of the lower limb buds of 12 mm embryos. In dissecting these out, I aimed to obtain only the femoral rudiments, but, as the results showed, in several instances part or whole of those of the tibia and fibula were included as well. The size and degree of differentiation of the femoral primordia implanted are shown in fig. 1, Plate 14. The hosts were fourteen baby rats, three to seven days old. They suffered no ill effects from the operation, and grew normally until they were killed nine weeks later. Twelve of the fourteen brains contained masses of bone, which in six cases took the form of recognizable long bones with epiphyses, while in the remaining six cases they were irregular and unanatomical in shape. The long bones obtained in the six successful cases were as follows:—(*a*) a slender bone 17 mm long with one well-formed epiphyseal end (figs. 2*a* and 3, Plate 14); (*b*) two incomplete long bones lying side by side, of total length 18 mm and with a joint and two epiphyses at the end of one fragment (fig. 2*b*, Plate 14); (*c*) the lower two-thirds of a femur 14 mm long, and a complete tibio-fibula 19 mm long, at an angle of 30° to one another, with well-formed epiphyses at their articulating ends where the knee-joint had undergone ankylosis by partial fusion of the two epiphyses (figs. 2*c* and 4, Plate 14); (*d*) and (*e*) slender bones 8 mm long, each with one well-formed articular end with epiphysis; (*f*) a femur 13 mm long with a distorted upper end and a well-formed lower end, articulating by a well-formed knee-joint with the normal upper end of the upper three-quarters of a tibia 12 mm long.

Three of the specimens, namely, (*d*), (*e*), and (*f*), after immersion whole in a dilute solution of alizarin sulphonate, showed the differential staining characteristic of growing bones, *i.e.*, the deepest staining took place at the epiphyseal ends of the diaphyses. Histological sections of the long bones obtained (figs. 3 and 4, Plate 14) showed that diaphyses and epiphyses were normal in shape and structure, and that epiphyseal growth was proceeding in a perfectly normal way. The medullary cavities contained normal haemopoietic marrow.

Remarks

In these experiments the implants consisted of undifferentiated cartilaginous rudiments about 1 mm long (fig. 1, Plate 14). After nine weeks

in the hosts' brains, these had grown into fully formed long bones up to 19 mm in length. This rate of growth is about the same as that of the corresponding bones in the intact animal; a healthy young rat two months old has a femur about 17 mm long and a tibia about 20 mm long.

The experiments show that the typical shape and structure of the bones, their mode and rate of growth, and the normal development of their articular ends and intervening joints, are all inherent in the potencies of the early cartilaginous rudiments and are not dependent on the nature of their surroundings. My results, therefore, confirm those of Fell and Robison (1929), whose cultivation of bone rudiments *in vitro* led to the same conclusions.

I am indebted to my wife for valuable help with the experiments, to Dr. C. A. Duncan for the skiagrams, and to Mr. Prosser, of the Alfred Hospital Pathology Department, for the microscopical sections.

Summary

The undifferentiated cartilaginous primordia of long bones from rat embryos, when implanted into the brains of young host rats, can grow at normal rates into the corresponding fully formed long bones. The capacities for growth and differentiation of long bones are, therefore, inherent in their cartilaginous primordia and not dependent on their surroundings.

References

Fell, H. B., and Robison, R. (1929). 'Biochem. J.,' vol. **23**, p. 767.
Willis, R. A. (1935). 'Proc. Roy. Soc.,' B, vol. **117**, p. 400.

Description of Plate 14

Fig. 1—A transverse horizontal section through a 12 mm rat embryo at the level of the lower limb bud. XX is the mid-line; L is the distal part of the limb bud; FF is the cartilaginous rudiment of the femur 1·2 mm long; PP is the pelvic rudiment with the acetabulum at its middle. Note that the femur is represented entirely by cartilage and mesenchymal pre-cartilage. × 35.

Fig. 2*a*, *b*, and *c*—Skiagrams of three of the hosts' brains containing the long bones described in the text. Note especially the epiphyseal ends visible in all three specimens, and the complete tibio-fibula in *c*. Natural size.

Fig. 3—Longitudinal section of part of the bone shown in fig. 2*a*. D is the diaphysis; E the epiphysis; and LL the epiphyseal zone of cartilage with characteristic vertical lines of cartilage cells undergoing ossification from the diaphyseal side. × 12.

Fig. 4—Longitudinal section of part of the bones shown in fig. 2*c*. The epiphyses EE of the femur F and the tibia T have partially fused across the plane of the knee-joint XX. × 12.

(*Abstract*)

541 . 123 . 127 : 545 . 81

Photoelectric Methods of Measuring the Velocity of Rapid Reactions

I—General Principles and Controls

By F. J. W. ROUGHTON and G. A. MILLIKAN

(From the Physiological Laboratory, Cambridge)

(*Received January* 22, 1936)

The need for an optical method of analysis of the moving fluid in a Hartridge-Roughton rapid reaction apparatus which shall be economical of fluid, non-subjective, and applicable to a wide range of colour changes, has been met by the application of photoelectric cells.

More accurate and comprehensive tests of both the efficiency of mixing and character of flow down the observation tube have been worked out.

As shown in Table III, fluid economy has been improved 10- to 100-fold. and the range of measurable reactions greatly extended as regards colour, without any loss in time range.

(*Abstract*)

541 . 123 . 127 : 545 . 81

Photoelectric Methods of Measuring the Velocity of Rapid Reactions

II—A Simple Apparatus for Rapid p_H and Other Changes, Requiring 200 cc or More of Each Reagent

By F. J. W. ROUGHTON

(From the Physiological Laboratory, Cambridge)

(*Received January* 22, 1936)

A simple photoelectric arrangement is described for analysing the colour of the streaming fluid in various parts of the observation tube of a Hartridge-Roughton apparatus.

The method has been applied especially to the colour changes of indicators, with the aid of which rapid p_H changes may be followed.

Under favourable conditions an accuracy of 0·01–0·02 p_H is reached, the distribution of the indicator between its two coloured forms being determinable to within about $\pm$ 1·0%.

The validity of the method for rapid reaction studies is supported by the reasonable agreement between values, as given by it, for the velocity constants of the reactions (i) $H_2CO_3 \rightarrow CO_2 + H_2O$; (ii) $CO_2 + OH' \rightarrow HCO_3'$, with the values of these constants as given by other methods.

The total amount of each reagent required for reactions of half-period $\geqslant$ 0·02 second is about 250 cc. With faster reactions more fluid would be required.

The application of the method to other colour changes is discussed.

(*Abstract*)

541 . 123 . 127 : 545 . 81

Photoelectric Methods of Measuring the Velocity of Rapid Reactions

III—A Portable Micro-Apparatus Applicable to an Extended Range of Reactions

By G. A. Millikan

(From the Physiological Laboratory, Cambridge)

(*Received January* 22, 1936)

The Hartridge-Roughton continuous flow apparatus for measuring rapid reactions has been modified so as to fit it for—

(*a*) reactions involving any kind of colour change (pigment reactions, and those involving indicators);

(*b*) small quantities of material;

(*c*) easy transport.

It has been used in the present form for several years in the study of small quantities of biological substances.

The reacting fluids are forced by motor-driven syringes through two converging tubes to a mixing chamber, whence they pass down the micro-observation tube, of approximately 1 mm internal diameter. The extent of the reaction is measured by means of a simple photoelectric cell colorimeter.

Control experiments checking the efficiency of mixing, the character of flow down the tube, and the overall performance of the apparatus, have shown it adequate to deal with processes whose half-reaction time is greater than about 0·0005 second.

When used for haemoglobin reactions, a complete kinetic curve can be obtained on 0·2 cc of blood (diluted about 200 times in the observation tube).

(*These papers are published in* '*Proc. Roy. Soc.*,' *A*, *vol.* 155, *pp.* 258, 269, 277 (1936).)

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